DIRECTED REORGANIZATION OF CHROMATIN TO THE NUCLEAR LAMINA IS MEDIATED BY CHROMATIN STATE, YING-YANG1 AND A-TYPE LAMINS

By

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Abstract

Nuclear organization has been implicated in regulating gene activity. Recently, large developmentally regulated regions of the genome that are dynamically associated with the nuclear lamina have been identified. However, little is known about how these lamina-associated domains (LADs) are directed to the nuclear lamina. We have generated genome-wide data that identifies lamina associated regions in desperate cell types, to study the organization of LADs. We utilize this data in combination with the development of the tagged chromosomal insert site system (TCIS) to discern the structural and functional relationships behind nuclear organization.

The TCIS system is comprised of a recombination cassette, to allow site-specific Cre-mediated recombination, as well as a *lacO* array that can reversibly bind EGFP-LacI for visualization of the locus. TCIS is then integrated in a single copy in a single location in fibroblast genome. Thus, TCIS enables integration of a single copy of any DNA fragment into the genome by directed recombination into the TCIS site and its visualization. Because any genomic element of interest can be specifically recombined into TCIS, this system is ideal for assaying association with a nuclear compartment of interest. This system makes it possible to study the mechanistic and functional consequences of genome organization at a single locus in a native state.

In this work we utilize TCIS to investigate the structural/functional relationships of the periphery as a nuclear sub-compartment. We have generated genome-wide data produced by DNA Adenine Methyltransferase Identification (Dam-ID) to identify small sequences from the borders of fibroblast-specific variable LADs that are sufficient to target these ectopic sites to the nuclear periphery. We identified YY1 (Ying-Yang1), CTCF, BTB-POZ domain protein binding sites (amongst others) as being enriched in relocating sequences. Knockdown of YY1 or lamin A/C, but not lamin A, led to a loss of

lamina association. In addition, targeted recruitment of YY1 proteins facilitated ectopic LAD formation dependent on histone H3 lysine 27 trimethylation and histone H3 lysine di- and tri-methylation. Our results also reveal that endogenous loci appear to be dependent on lamin A/C, YY1, H3K27me3, and H3K9me2/3 for maintenance of lamina-proximal positioning.

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Abbreviations and Acronyms

A	adenine
BAC	bacterial artificial chromosome
BAF	barrier to autointegration factor
bcl11a	B-cell CLL/lymphoma 11A
Bleo	bleomycin
BTB/POZ	BTB (for BR-C, ttk and bab) or POZ (for Pox virus and Zinc finger)
BiFC	bimolecular fluorescence complementation
bp	base pair
ChAP-MS	chromatin Immunoprecipitation- Mass Spectroscopy
ChIA-PET	chromatin interaction analysis by paired-end tag sequencing
ChIP	chromatin Immunoprecipitation
C	cytosine
CBX	chromobox Homolog
cDNA	complementary DNA
CFTR	cystic fibrosis gene
CFP	cyan fluorescent protein
cLAD	common Lamina associated domain
CMV	cytomegalo virus
CO_2	carbon dioxide
CTCF	CCCTC-binding factor
CFP	cyan fluorescent protein
Dam	DNA adenine methyltransferase
DamID	DNA adenine methyltransferase Identification
DMEM	Dulbecco's Modified Eagle's medium
DNA	deoxyribonucleic acid
Dnmt3a	DNA Methyltransferase
DZNep	3-deazaneplanocin
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
FISH	fluorescence in situ hybridization
EGFP	enhanced green fluorescent protein
EMD	Emerin
ESC	Embryonic Stem Cells
EZH2	enhancer of zeste 2
FB	fibroblast
G	guanine
gDNA	genomic DNA
GFP	green fluorescent protein
GCL	germ-cell-less
G9a	histone-lysine N-methyltransferase, H3 lysine-9 specific 3
H3	histone H3
H4	histone H4
HAT	histone acetyltransferases
HDAC	histone deacetylases
HDM	histone demetyhlases
HMT	histone methyltransferases
HP1α	heterochromatin protein 1 alpha

Hyg	hygromycin
IgK	Immunoglobulin kappa locus
Izkf1/Ik	Ikaros
IF	immunofluorescence
Igh	immunoglobulin heavy chain locus
INM	inner nuclear membrane
IPTG	isopropyl b-D-1-thiogalactopyranoside
K	lysine
kb	kilo base
L1/1L	LoxP site
LacI	LacI repressor protein
*lac*O	*lac* Operator
LAD	lamina associated domain
LAP	Lamina associated peptide
LAS	lamina associated sequence
LBR	lamin B receptor
LiCl	lithium chloride
LMNA	Lamin A
LMNA/C	Lamin A and C
LMNC	Lamin C
LMNB1	Lamin B1
L2β	Lap2Beta/ Lamina associated peptide-2/Beta
Mb	mega base
me	methyl
MEF	mouse embryonic fibroblast
NaCl	sodium chloride
NPC	nuclear pore complex
ONM	outer nuclear membrane
PBS	phosphate buffered saline
PcG	polycomb group
pen	penicillin
PCR	polymerase chain reaction
PML	promyelocytic leukemia
PMT	post translational modification
PRC1	polycomb repressive complex 1
PRC2	polycomb repressive complex 2
qPCR	quantitative PCR
RAG	Recombination Activating Gene
Rb	retinoblastoma protein
RMCE	recombination mediated cassette exchange
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SSC	saline-sodium citrate
strep	streptomycin
T	thymine
TCIS	tagged chromosomal insertions site
tetO	tetracycline Operator
tetR	tetracycline Repressor
TK	Thymidne Kinase
vLAD	variable lamina associated domain

YFP	yellow fluorescent protein
YY1	Ying-Yang-1
Zbtb7b	cKrox/TH-Pox
2D	two-dimensional
3D	three-dimensional
3C	chromosome conformation capture
4D	four-dimensional

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Chapter One

Introduction

1.1: Gene regulation and chromatin organization

Developmentally regulated genes become activated or repressed by a variety of mechanisms involving both local and global changes. Local changes include direct interactions of *trans* factors (i.e. cell/developmentally specific transcription factors) or *cis* factors in the DNA (i.e. promotors and regulatory elements). In eukaryotic cells, DNA is organized into nucleosomes containing 146 bases of DNA wrapped 1.65 times around a histone protein octamer consisting of two H2A and B heterodimers and a histone H3/H4 tetramer (Luger, Mäder, Richmond, Sargent, & Richmond, 1997). These nucleosomes are then packaged into chromatin (DNA plus histones with associated proteins and complexes) which serves as the substrate for virtually all nuclear events (transcription, replication, recombination, and repair). Modulation of transcription (and other DNA-based activities) is influenced by changes in the post-translational modifications of histone tails, DNA methylation patterns and/or nucleosome positioning. In particular, such modifications to chromatin impact the ability of specific transcription factors to interact with their cognate binding site(s) and conversely, binding of a given transcription factor to its binding site can alter local chromatin structure, thus tightly linking transcription of a gene with its chromatin state. There are two broad 'types' of chromatin: heterochromatin which is more densely packed and thus repressive to gene activity and euchromatin which is more open and permissive to gene expression (Figure 1.1). Because such modifications to the chromatin influence gene expression and are heritable through the cell-cycle, this type of control is called 'epigenetic' regulation. Intriguingly, more recent evidence strongly suggests that there is another more global level of epigenetic regulation, involving changes in nuclear localization, associations with repressive compartments, such as the nuclear periphery or pericentromeric heterochromatin, and large-scale changes in DNA structure, such as the formation of DNA loops and/or locus contraction. However, it remains unclear how this three-

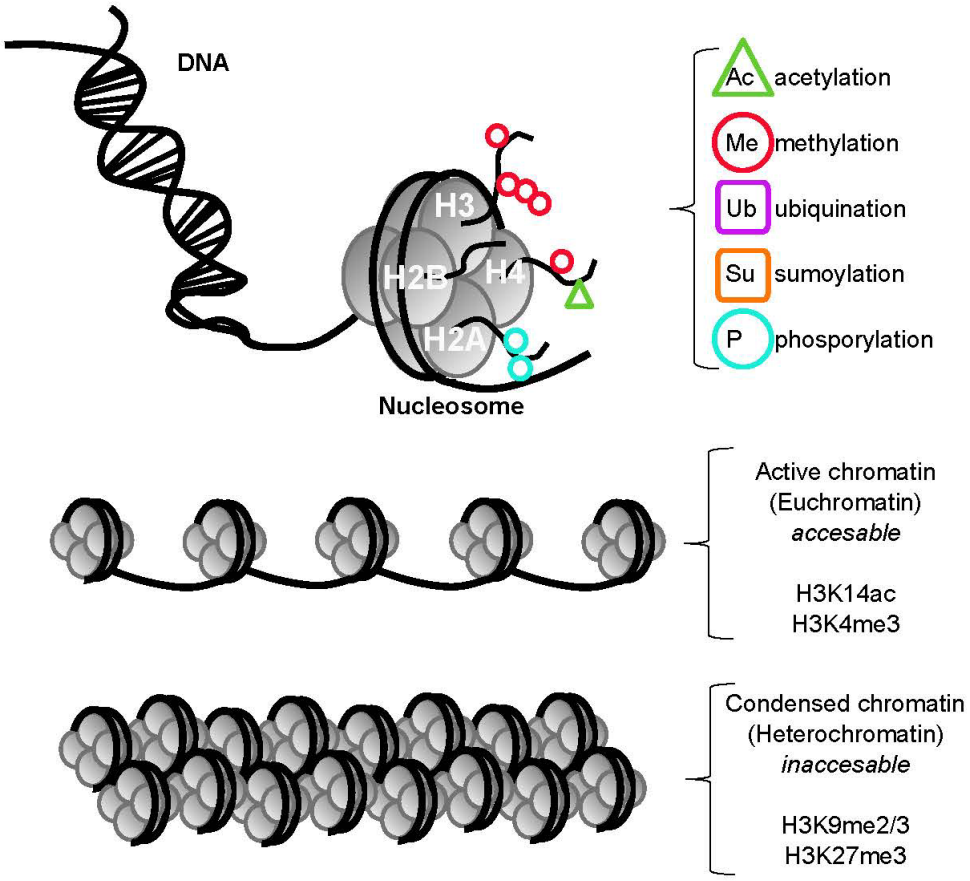


Figure 1.1: Chromatin organization. Chromatin is comprised of DNA, histones and associated proteins. The basic unit of chromatin is the nucleosome. The nucleosome is a histone octamer (Histone H3/H4 tetramer, and two H2A and B heterodimers) wrapped 1.65 times by 147bp of DNA. Post translational modifications (PTMs) on histone tails, decorate the outside of the nucleosome. Common histone modifications include methylation, acetylation, ubiquitination, sumoylation and phosphorylation. In general there are two forms of chromatin, a more open form in which transcription factors and proteins can interact with DNA, (euchromatin) and a more closed structure where the DNA is less accessible (heterochromatin). Euchromatin is generally permissive to transcription and is commonly decorated with acetylation of H3 and H4. In contrast, heterochromatin is generally repressed and contains, H3K27me3 and H3K9me2/3. This basic level of chromatin organization underlies a more complex interplay of chromatin with in subdomains in the nucleus which appear to organize DNA and regulatory factors that in turn lead to an overall dynamic control over development and cell state.

dimensional organization changes during development or upon environmental cues and, more importantly, how these changes may impact genome/epigenome regulation.

1.2: The nuclear periphery and chromosome organization

The nuclear periphery in metazoan cells is composed of a unique set of inner nuclear membrane (INM) proteins and the nuclear lamina (Verstraeten, Broers, Ramaekers, & van Steensel, 2007). Mammalian INM proteins include, but are not limited to, lamin B receptor (LBR), lamina associated peptide2 (Lap2) and Emerin (Wilson & Berk, 2010). These and other INM proteins interact with the nuclear lamina, which is made up of a filamentous meshwork of proteins: lamins A/C and B (Gerace & Burke, 1988; Moir, Montag-Lowy, & Goldman, 1994; Stuurman, Heins, & Aebi, 1998). LBR, Emerin, and Lap2, for example, have been shown to interact with transcriptional repressors. Specifically, LBR has been shown to interact with heterochromatin protein 1α (HP1α) and nucleosomes through the core histones H3 and H4 and the LEM (Lap2β, Emerin, MAN1) domain containing proteins, Emerin and Lap2β, interact with Barrier to Auto-integration Factor (BAF), germ-cell-less (GCL), retinoblastoma protein (Rb) and HDAC3 (Holaska & Wilson, 2007; Kourmouli et al., 2000; Somech et al., 2005; Ye, Callebaut, Pezhman, Courvalin, & Worman, 1997; Ye & Worman, 1996). These studies suggest a strong role for the INM in genome regulation.

A growing body of evidence suggests that the proteome at the INM is quite complex, containing over 100 different and differentially expressed transmembrane proteins, many of which are developmentally regulated or variably expressed across cell types (Florens, Korfali, & Schirmer, 2008; Korfali et al., 2010; Malik et al., 2010; Schirmer & Foisner, 2007; Wilkie & Schirmer, 2008; Wilson & Berk, 2010; Wilson & Foisner, 2010). High throughput proteomics approaches that assay for all nuclear envelope transmembrane (NET) proteins have recently described numerous proteins

that vary depending upon cell type. These proteins include both well-known and novel INM proteins (Korfali et al., 2012). It has been demonstrated that these tissue and cell type specific NETs contribute to cell type specific peripheral localization of whole chromosomes and, potentially, to cell type specific gene regulation (Zuleger et al., 2013).

An example of such differentially expressed proteins playing a role in genome regulation and organization is LBR. Even before these more recent studies using advanced proteomics tools, the level of LBR was known to affect genome organization and developmental progression in certain tissues (Gaines et al., 2008; A L Olins, Herrmann, Lichter, & Olins, 2000; A. Olins & Olins, 2004; Solovei et al., 2013; Zwerger, Herrmann, Gaines, Olins, & Olins, 2008). LBR has multifunctional roles at the INM, including the promotion of membrane growth and regulation of genome organization by its ability to bind to epigenetic factors (e. g histones and HP1) and B type lamins (Gaines et al., 2008; Ada L Olins, Rhodes, Welch, Zwerger, & Olins; Zwerger et al., 2008). The expression levels of LBR are correlated with the degree of segmentation or hyper-lobulation of granulocyte nuclei, proper heterochromatin organization and neutrophil development during granulopoeisis, as promyelocytes (or earlier precursors) differentiate into granulocytes. Furthermore, both LBR and lamins A and C have been implicated in recruiting or scaffolding heterochromatin at the nuclear periphery (Gaines et al., 2008; A L Olins et al., 2000; A. Olins & Olins, 2004; Solovei et al., 2013; Zwerger et al., 2008). Additionally, when cells are devoid of both LBR and lamin A/C they exhibit an inverted chromatin configuration similar to that seen in rod cells of nocturnal animals that naturally lack LBR and lamin A/C expression (Solovei et al., 2009, 2013). This inverted configuration displays heterochromatin occupancy in the nuclear interior while euchromatin has a more peripheral association. This organization is critical for diminishing light scattering and hence allowing vision under low light conditions (Solovei et al., 2009). Interestingly, while LBR is sufficient in preventing this inversion, ectopic

expression of lamin C in rod cells did not, suggesting the requirement of mediator(s) for anchoring chromatin to the nuclear periphery (Solovei et al., 2013). It is currently unclear exactly which of the A-type lamins are responsible for maintaining normal scaffolding of heterochromatin to the lamina.

1.3: The chromatin compartment at the nuclear periphery-Lamina Associated Domains (LADs)

Early cytological studies using electron-microscopy clearly show heterochromatin enriched at the nuclear periphery, suggesting that this region of the nucleus acts as a compartment that contains inactive regions of DNA. A natural hypothesis to emerge from these observations is that chromatin in this regions will harbor epigenetic characteristics of heterochromatin. In recent years, the DNA Adenine Methyltransferase Identification (DamID) technique has been used to map genome-wide chromatin contacts at the nuclear periphery to identify lamina associated domains (LADs) of DNA (Vogel, Peric-Hupkes, & van Steensel, 2007 and see Chapter 2 for a more detailed discription of the technique). Initial reports characterized LADs as large (0. 1-10Mb) silent domains with low gene density or 'gene deserts' (Guelen et al., 2008). More recent studies, and the work herein, have focused instead on LADs that do contain genes. Studying this subset of LADs enables the exploration of the function such organization may play in gene regulation (Reddy, Zullo, Bertolino, & Singh, 2008; Zullo et al., 2012). In particular, developmentally regulated genes appear to be enriched in these domains, leading to the hypothesis that LADs serve as 'facultative' heterochromatin domains during development (Meuleman et al., 2013; Peric-Hupkes et al., 2010). Additionally, while LADs are mostly composed of heterochromatin (see below), it is important to note that there are several examples of lamina-proximal genes that are actively transcribed (Brickner et al., 2012; Kumaran & Spector, 2008; Yao, Fetter, Hu, Betzig, & Tjian, 2011).

These genes may interact with nuclear pore complexes, which have been shown to reside in a euchromatic environment, perhaps to both facilitate transcriptional activation and transport mRNA into the cytoplasm (Brickner et al., 2012). However, the large domains of chromatin that come in molecular contact with the nuclear lamina are repressed and it is these inactive, but gene-containing, LADs that are an active area of research for our group—specifically to uncover their role in chromosome architecture and gene regulation.

DamID studies in our lab and others' have implicated LADs in both epigenetic modulation and control of gene expression. These studies rely on intersecting DamID data with genome-wide chromatin immunoprecipitation (ChIP) analyses used to detect epigenetic modifications and transcriptional regulators across the genome (and with high resolution). Specifically, it has been noted that LAD borders, but not the interior of these domains, are enriched in the facultative heterochromatin histone H3 lysine 27 trimethylation (H3K27me3) (Guelen et al., 2008; Zullo et al., 2012, Shah et al., 2013). In this original study, a modest but not impressive correlation with Histone lysine 9 tri-methylation (H3K9me3) was noted, and similar ChIP experiments for the histone H3 lysine 9 di- or tri-methylation (H3K9me2/3) modification shows a domain profile similar to LAD in size, distribution and position (Bo Wen, Wu, Shinkai, Irizarry, & Feinberg, 2009). When these large organized chromatin K9 modifications (LOCKs) were compared to available LAD data, an approximately 80% overlap was noted. However, these assays were done in disparate cell types, so the extent of LAD/LOCK is currently unknown. Nonetheless, it appears that these two heterochromatin marks, H3K27me3 and H3K9me2/3, are correlated with LADs. We note, however, that H3K27me3, unlike H3K9me2/3, is found *mostly* outside of lamina proximal domains, with a higher than expected enrichment at LAD borders (Guelen et al., 2008 and see Chapter 2). Our work aims to determine the dynamic and causal relationship between LADs and chromatin

state, i.e. if LAD association is dependent upon chromatin state or vice versa (or both). Several recent studies have implicated chromatin status in directing LAD organization. A modified live-cell DamID protocol, using a highly clonal cancer cell line, showed that reduction in G9a, the methyltransferase that deposits H3K9me3 modifications, led to a 'loosening' of LAD structure near the nuclear periphery (Kind et al., 2013). Additionally, a series of experiments in *C. elegans* pointed to the role for H3K9me2 in targeting to the periphery and subsequent accumulation of H3K9me3 in these domains after becoming lamina proximal (Towbin et al., 2012). However, in embryonic stem cells (ESCs), profound differences in these domains were observed with LOCKs being largely absent and LADs displaying robust configuration (Peric-Hupkes et al., 2010; Bo Wen et al., 2009). Interestingly, the genomes of ESCs demonstrate a unique overall 3D organization (de Wit et al., 2013). Given these data we propose that epigenetic state and LAD organization are inter-related, and we describe in chapter two how these modifications are required for formation and/or maintenance of lamina associated domains.

Given the studies implicating chromatin state in directing compartmentalization, it is important to note that forced localization of genes to the inner nuclear membrane leads to their repression, indicating that this compartment may also contribute to epigenetic changes (Finlan et al., 2008; Kumaran & Spector, 2008; Reddy et al., 2008). These functional experiments are consistent with numerous genome-wide and cytological studies examining lamina proximal chromatin (Kosak et al., 2002; Meister, Towbin, Pike, Ponti, & Gasser, 2010; Peric-Hupkes et al., 2010; Williams et al., 2006; Yao et al., 2011; Zullo et al., 2012). Taken together these data strongly suggest a role for the nuclear periphery as a compartment that functions in the dynamic establishment and/or maintenance of gene repression. A rapidly growing focus of research is on the developmentally and cell type specific dynamic interaction of the genome and epigenome with the nuclear lamina and the peripheral zone.

1.4 Additional features of LADs and LAD borders

Additional analyses of genomic features in the context of lamina associated domains have revealed a few characteristics that further suggest functionality and dynamics of the chromatin present at the nuclear periphery. Consistent with gene-poor and repressed chromatin, LINE elements and A/T sequences are enriched in LADs, as are promoters in adjacent domains that drive transcription away from LAD border regions, perhaps contributing to the delineation of the borders (Meuleman et al., 2013). It is interesting to note that H3K9me2/3 domains that correlate highly with LADs have "islands of euchromatin" within a larger LOCK domain, as evidenced by low H3K9me2/3 signal, and these islands overlap with transcription start sites (B Wen et al., 2012). We note these same breaks in LAD organization, suggesting a link between H3K9me2/3, transcription and LAD organization over these regions. The potential functional role of these regions is unknown, but they may be indicative of regulatory regions important to LAD dynamics and/or may have a role in scaffolding.

In addition to the H3K27me3 modifications noted above, the borders of LADs appear to be enriched in binding sites for the chromatin organizer CTCF (CCCTC-binding factor) (Dixon et al., 2012; Guelen et al., 2008; Handoko et al., 2011; Nora et al., 2012; Zullo et al., 2012). CTCF is a highly conserved zinc finger DNA binding protein that acts as an insulator and has also been shown to interact with DNA in large and varied protein complexes to facilitate long range inter- and intra- chromosomal interactions (Van Bortle & Corces, 2013). CTCF is also involved in higher order organization of the genome into topological domains, also known as Topologically Associating Domains or TADs (Dixon et al., 2012; Li et al., 2013; Nora et al., 2012; Phillips-Cremins et al., 2013; Sexton et al., 2012). TADs are large domains of the genome, detected by chromosome conformation capture derivative methods (5C or Hi-C) which preferentially interact within a single domain and exhibit very few interactions

with adjacent TADs (Dixon et al., 2012). Intriguingly, LADS and TADs share boundaries with a much higher than expected frequency than expected by chance, but many LADs cross topological domain boundaries and vice versa, suggesting that these structures are not identical. (Dixon et al., 2012; Nora et al., 2012; Phillips-Cremins et al., 2013). In addition, LADs are quite dynamic across cell types, while TADs are much less so, with sub-domains within TADs showing much greater changes between cell types.

Interestingly, CTCF has been proposed to interact with Ying-Yang1 (YY1) to mediate sub-TAD cell type–specific organization, raising the possibility that CTCF and YY1 act together to target and delimit LAD boundaries (Phillips-Cremins et al., 2013). Numerous studies have implicated the interaction of these two proteins in both nuclear architecture and in gene regulation (Donohoe et al., 2007; Kim, 2008; Nikolaev et al., 2009; Degner et al., 2011; Weth and Renkawitz, 2011; Medvedovic et al., 2013; Wei et al., 2013). YY1 itself is a general transcription factor that has been implicated in both activation and repression of specific genes (Deng et al., 2010). Intriguingly, YY1 has been speculated to mediate gene repression through recruitment of the polycomb repressive complex (PRC2), which introduces H3K27me3 through the activity of EZH2 (Enhancer of Zeste 2), a component of PRC2 (Atchison, Ghias, Wilkinson, Bonini, & Atchison, 2003; Basu, Wilkinson, Colavita, Fennelly, & Atchison, 2014; Srinivasan & Atchison, 2004; Wilkinson, Pratt, & Atchison, 2010). In mammalian cells it is unclear how PRC2 is recruited to DNA. In drosophila, YY1 has been shown to interact with the EED component of YY1 (Atchison, Ghias, Wilkinson, Bonini, & Atchison, 2003; Caretti, Di Padova, Micales, Lyons, & Sartorelli, 2004; Meara & Simon, 2012; Satijn, Hamer, den Blaauwen, & Otte, 2001). As previously mentioned, the chromatin insulator protein CTCF has been shown to have its cell-type specific functions, for example in X-chromosome inactivation, modulated by neighboring DNA binding factors, such as YY1 (Degner et al., 2011; Donohoe, Zhang, Xu, Shi, & Lee, 2007; Lin et al., 2012; Van Bortle

& Corces, 2013; Zlatanova & Caiafa, 2009) To date, no direct role for H3K27me3, CTCF or YY1 in LAD organization has been elucidated.

1.5: Three dimensional regulation of the immunoglobulin heavy chain locus

For proper coordination with lymphocyte differentiation, the somatic rearrangement of lymphocyte receptor loci must be lineage and developmental stage specific. However, the RAG (Recombination Activating Gene) recombinases, which enable the somatic rearrangement of the antigen receptors, are expressed in B and T cells and their progenitors, and other factors involved in V(D)J recombination are ubiquitous (e.g. factors involved in nonhomologous end joining). Specificity of this process is therefore guaranteed only through cell-type specific transcriptional and epigenetic environments, making the antigen receptor genes model loci for understanding how key developmentally regulated genes are controlled. Multiple developmentally regulated mechanisms appear to be responsible for relieving repression of the Igh loci as cells are specified to the B cell lineage. In non-B cells and progenitor cells the heavy-chain locus is found in a repressed state (H3K9 methylated, hypo-acetylated) at the nuclear periphery. Early in B cell development, key transcriptional regulators such as E2A (E2a immunoglobulin enhance binding factors E12/E47, aka TCF3 or transcription factor 3) and EBF (early B cell factor) are upregulated (K L Medina & Singh, 2005). This coincides with the rearrangement and transcription of the DJ H cluster, which displays increased histone acetylation. Following DJ H rearrangement, the proximal portion of the V H locus becomes transcriptionally and recombinationally activated. This activation is accompanied by increases in histone acetylation levels in this region of the locus. Concomitantly, the heavy-chain locus becomes localized to a more centralized location (i.e. moves away from the nuclear lamina) in the nucleus via as yet unidentified factors and mechanisms (Johnson et al., 2008; Kosak et al., 2002;

Reddy et al., 2008). Cells are now at the proB cell stage and continued development and survival is dependent on the cytokine interleukin-7 (IL-7) (Carvalho, Mota-Santos, Cumano, Demengeot, & Vieira, 2001; Dias, H Jr., Cumano, & Vieira, 2005; Miller et al., 2002). IL-7 signaling activates Stat5 (signal transducer and activator of transcription 5), which induces increased histone acetylation at the distal portion of the V H locus leading to transcriptional upregulation and recombination to these segments (Bertolino et al., 2005; Kosak et al., 2002; Reddy et al., 2008). Pax5 (paired box 5) and Ezh2 (enhancer of zeste 2) are also upregulated at the proB cell stage. Pax5 and Ezh2 regulate histone methylation of H3K9 (histone H3, lyine 9) and H3K27 (Histone H3, lysine 27), respectively, leading to the activation of the locus (Johnson et al., 2004; Su et al., 2003). Pax5 and Ying Yang 1 (YY1) also contributes to more global changes at the locus leading to long-range locus contraction with another unidentified B cell factor (Fuxa et al., 2004). Collectively, these changes allow V-to-DJ H recombination and commitment to the B cell lineage. Though many questions remain, it is clear that the same signals that drive B cell development exert their control over heavy-chain recombination; thereby ensuring that recombination of the immunoglobulin locus is both lineage specific and developmentally regulated. Importantly, these same transcriptional regulators also impact the three dimensional organization and architecture of the Igh locus.

Specifically, it has been shown by us and others, using 3D-ImmunoFISH, that germ-line immunoglobulin heavy chain loci (*Igh*) are preferentially localized to the nuclear lamina in hematopoietic progenitors, T lineage cells and non-B cells (such as fibroblasts) but centrally positioned in proB cells, where they are active (Kosak et al., 2002; Reddy et al., 2008; Yang, Riblet, & Schildkraut, 2005; Zhou, Ermakova, Riblet, Birshtein, & Schildkraut, 2002). Importantly, the inactive, lamina-proximal, Igh alleles in T cells do not co-localize with pericentromeric heterochromatin. Reddy et al, hypothesized that the preferential association of the variable (V_H) gene segments of Igh loci with the

nuclear periphery in non-B lineage cells renders them inactive for transcription and DNA recombination (Reddy et al., 2008). Additional studies have revealed an exquisite three dimensional choreography of the immunoglobulin loci during B cell development. Using mutations for transcription factors necessary for B cell development, it has become evident that not only is transcriptional activation/regulation of these loci dependent upon key developmental regulators, but so is the positioning of the Ig loci relative to different nuclear compartments (specifically the nuclear lamina/INM and centromeric heterochromatin). It however, remains largely unknown what factor(s) mediate repositioning of the Ig loci and if it's dynamic organization to different nuclear compartments serves a regulatory role. Moreover, while the Igh locus is relatively well studied, virtually nothing is known about other important loci are regulated in a similar manner during B cell development.

As mentioned previously, analyses of LADs domains revealed that they are enriched in silent genes (Guelen et al., 2008). Indeed, the entire 3 megabase Igh locus comprises an entire LAD in cell types in which the locus is inactive (i.e. fibroblasts) and is non-lamina associated in pro-B cells when the locus is active. We and others have uncovered numerous other LADs that change upon developmental progression (Zullo et al., 2012, Harr et al., 2015; Peric-Hupkes et al., 2010). We have named these LADS that change their configuration between cell stages or cell types vLADs for 'variable' Lamin Associated Domains. While initial studies have been done to map the genome-wide association of these vLADs regions with changes in epigenetic and transcriptional states, to truly assay the functional regions and consequences of nuclear scaffolding an assay must be developed to test these correlative studies (Peric-Hupkes et al., 2010). Given that the majority of LADs remain the same from cell type to cell type, it is imperative to focus on those regions that change upon developmental progression to understand the role that nuclear dynamics is playing. It is unclear how dynamic

association/dissociation of chromosomal domains from LADs impacts chromatin or transcriptional status of genes within those regions. While the entire Igh LAD is lost from pro B cells to fibroblasts, in most cases we note that variable LADs are a loss of the border regions, where the edge of the LAD appear to peel back and reveal the developmentally regulated genes (see Chapter 2 for more detail).

1.6: Functional and dynamic organization of LADs in development and cellular function

The *Igh* locus and other developmentally regulated genes that display variable lamina association dependent on cell state lead us to hypothesize that the nuclear periphery can act as a functional compartment to specifically regulate gene activity. What is not known is how these genes and other regions are recruited or directed to this region of the nucleus and their properties once resident there. When deciphering information gained by cytological and genome-wide studies, it is important to note that it is unknown whether LADs identified by DamID or other means are actually scaffolded at the INM/lamina or whether they represent a population sequestered to a more peripheral region. As previously mentioned, recent live cell studies in cancerous cell lines demonstrated that LADs are dramatically and dynamically repositioned after transiting the cell cycle, even well away from lamina-proximal positions (Kind et al., 2013). Given the experimental limitations of these techniques (overexpression of the Dam-LMNB1 fusion protein and the use of a highly transformed cell line), it is unclear whether these long range and apparently stochastic movements are a property of normal LAD organization and dynamics. Aside from these limitations, these studies uncovered an interesting phenomenon occurring at the nuclear periphery—an apparent stochasticism and dynamics within what we have termed, the ‘peripheral zone’. Studies in developing myoblasts which identified the endogenous *Myod1* gene as localized to the periphery

and expressed at very low levels (Yao et al., 2011), support the concept of a peripheral zone. In these studies, the MyoD locus was retained in the peripheral region until activation/differentiation. Specific exclusion of the core transcription factor TAF3 away from the zone at the periphery was demonstrated to be important for the relative inactivity of the *MyoD1* locus in early myogenesis and H3K9me3 was demonstrated to be enriched in this same zone. Interestingly, artificially tethering a *Myod1* promoter transgene to the nuclear periphery results in total transcriptional repression of the transgene, raising the provocative possibility that protein-lamina tethering and localization to the peripheral zone both serve to regulate gene activity, but to differing degrees (Yao et al., 2011). As mentioned previously, numerous other studies have shown interactions of chromatin modifiers and scaffolding proteins at the periphery, for example HDAC3 interactions with Lap2β, HP1 with LBR and BAF with numerous INM proteins and chromatin (Berk, Tifft, & Wilson; Ada L Olins et al.; Zullo et al., 2012). As demonstrated by the regulation of the *MyoD1* gene and as discussed previously for the differential roles of LBR (See, 1.1: The nuclear periphery and chromosome organization), compositional changes in the IMN protein and or nuclear lamina network likely have effects on gene regulation and chromatin scaffolding.

This raises the question of how a genic region might be directed to or away from, the nuclear periphery. While evidence supports that gene activation and movement away from the nuclear periphery are coupled, it is currently unclear whether activation precedes movement away from this repressive domain, and this is an area of current investigation (Hübner, Eckersley-Maslin, & Spector, 2013; Kumaran & Spector, 2008; Noordermeer et al., 2011; Peric-Hupkes et al., 2010). No LAD ‘zipcode’ has yet been identified and it is important to note that there are different types of LADs—those that change their LAD status upon differentiation or cell state changes (vLADs or variable LADs) and those that appear to remain constant amongst several cell types (cLADs or

constant LADs). It is evident that cLADs and vLADs exhibit different responses to developmental cues and, thus, not all LADs are equal. Additionally, it is in the vLAD regions that we see an enrichment of developmentally regulated genes, and we therefore hypothesize that these regions play a special role in gene regulation. It will therefore be important to analyze vLADs specifically to uncover sequences and mechanisms that allow for regulated and directed reorganization of genic regions in a cell type specific manner. As an example, a collaborative study in our lab focused on the *Igh* and *Cyp3a* loci which are lamina-proximal in murine fibroblasts but nucleoplasmic in both pro-B cells and hepatocytes (Zullo et al., 2012). In these studies, Lamina Associated Sequences (LASes) from these vLAD regions were identified that were able to direct repositioning of ectopic regions to the nuclear periphery when randomly (and iteratively) integrated into the genome of fibroblasts—a cell type in which the endogenous genes are normally inactive and lamina-proximal (Zullo et al., 2012). These iterated copies of LASs can be thought of as potential lamina 'zipcodes'. The LAS sequences identified were enriched in GA di-nucleotides, which is striking given that LAD domains are relatively A/T rich. It is tempting to speculate that such LAS sequences may be important for establishing peripheral compartmentalization of genomic regions during cellular differentiation. It is, however, still unclear how decisions pertaining to cell type specific LADs are being made given an (almost) identical genome across cell types of an organism. It will be necessary and we aim to uncover more such LAS elements from vLADs, to determine whether such sequences are truly sufficient and what proteins mediate reorganization into a *de novo* LAD.

The above mentioned work also uncovered a role for the cell type specific transcription factor (Zbtb7b), a histone deacetylase (HDAC3) and an INM protein (LAP2β) in reorganizing genic regions to the nuclear periphery. Zbtb7b encodes a BTB

POZ domain protein that recognizes $GA_{(n)}$ sequences and can act as both a transcriptional repressor or activator (Melnick et al., 2002). Another BTB-POZ domain protein, mGCL (murine Germ-Cell-Less, also a transcriptional repressor) interacts with Lap2β and Emerin in the INM, suggesting the intriguing possibility that this large family of differentially and developmentally expressed proteins may play a role in LAD architecture and function (Kimura et al., 2003). In addition, the BTB-POZ domain has been shown to recruit PRC2 to chromatin. As previously mentioned YY1 also can interact with PRC2, mediating a similar event, leading to the possibility that that targeting of LASes by these proteins leads to H3K27me3 as well as association with the nuclear periphery (Boulay et al., 2012). In further support for this model, in *Drosophila* neurons, the neural competence factor hunchback (*Hb*) becomes repressed and lamina-proximal concurrently with down-regulation of Distal Antenna (Dan) (Kohwi, Hiebert, & Doe, 2011). Dan contains a 'pipsqueak' GAGA DNA binding motif that competes with BTB-POZ domain proteins for its target sites, and expression of Dan prevents the lamina-proximal positioning and repression of *Hb* (Lehmann, Siegmund, Lintermann, & Korge, 1998). It is hypothesized that LAS containing regions recruit specific factors (e. g. Zbtb7b or Dan) that in turn either promote (Zbtb7b) or inhibit (Dan) association with the nuclear periphery. In addition, these cell type specific transcription factors could recruit chromatin remodelers [or interact with proteins, such as YY1 that recruit modifiers (PRC2), see Chapter 2 for further discussion] for the induction of large scale chromatin changes concurrent with either localization to or away from the nuclear periphery. Moreover, epigenetic state may be the overriding factor in the 'decision' to be relegated to the peripheral zone and LAS sequences with specific protein partners may merely act as a molecular switch for inducing a specific chromatin signature that drives the reorganization of specific regions to the repressive nuclear periphery. In either case, it appears that combinatorial information (protein binding or epigenetic state, for instance)

on top of the LAS sequences is needed to discriminate between domains that remain nucleoplasmic and those that are destined to become LADs in a cell type specific manner. It is likely that more cell type specific factors (both the previously mentioned NETs and transcriptional regulators) will be identified in regulating the dynamic LAD landscape, both in development and disease.

1.7: Imaging of Nuclear Dynamics

1.7.1: Insights into Chromosome Dynamics Using Live Cells: The LacO System

As outlined above, the search for the mechanisms underlying nonrandom chromosome and gene organization within the nucleus, particularly scaffolding at the nuclear lamina, as well as **t**he functional consequences of such a configuration lies at the heart of research into nuclear dynamics. In order to determine such structure/function relationships, a manipulable live cell synthetic genomic locus would be the ideal test system for such studies. The LacI/*lacO* system has been widely used in mammalian cells to study chromosomal dynamics and chromatin decondensation. These arrays can also be used to visualize a chromosomal domain and its movements in living cells under many conditions (Figure 1.2). Previous studies have demonstrated that LacI binds to *lacO* sites with high affinity (Kd = 10^{-10}) and DNA binding is reversed upon incubation with the allosteric inhibitor isopropyl b-D-1-thiogalactopyranoside [IPTG (Cronin, Gluba, & Scrable, 2001; Dong et al., 1999)]. Importantly, in mammalian cells a fluorescently tagged LacI protein is able to bind to integrated *lacO* sites (256 copies) specifically and can be visualized using simple live cell microscopy techniques (Belmont, Li, Sudlow, & Robinett, 1999). Thus, this system provides for highly specific and stable DNA binding that is reversible using a relatively inexpensive reagent (IPTG). These artificially designed chromosomal domains have been used to demonstrate *in vivo*

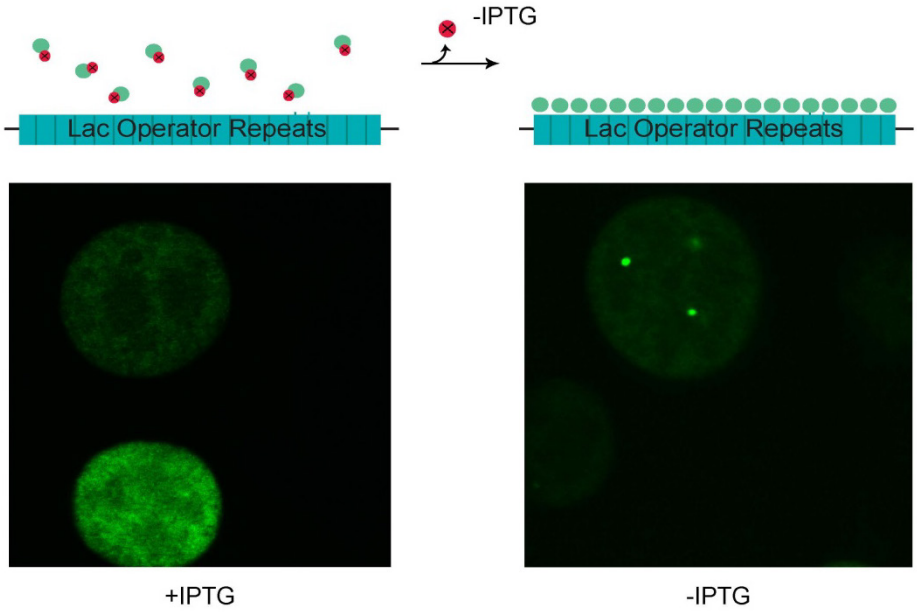


Figure 1.2: Stably integrated arrays of *lacO* binding sites are visible upon binding of GFP-LacI in living cells. Shown are cells containing the *lacO* repeats that are also expressing GFP-LacI. This interaction is controlled by the allosteric inhibitor IPTG. IPTG can be used in the culture medium to prevent GFP-LacI from binding (+IPTG). Upon IPTG withdrawal (IPTG), accumulation of the GFP-LacI protein on the *lacO* arrays occurs in a matter of hours. This system has been widely used to look at chromosomal domain dynamics (several megabases) with large amplified regions of *lacO* repeats or dynamics of smaller regions (>10 kb) using single integrations.

dynamics of large amplified arrays of *lacO* binding sites occupying megabases of DNA as well as more discreet regions comprising only one or two sites of integration (10 kb; (Belmont et al., 1999). These integrated arrays have been demonstrated to exhibit simple Brownian motion in interphase nuclei and the larger (Mb) sized integrations, are quite often condensed and heterochromatic (Belmont et al., 1999; Tumbar, Sudlow, & Belmont, 1999). Upon binding of a transcriptional activator to these large designer chromosomal domains, the decondensation of the domain is dramatic and easily seen by light microscopy (Tumbar et al., 1999). In addition, a random integration positioned reproducibly at the nuclear periphery shows dramatic relocalization to the nuclear interior upon trans-activation along with rapid and apparently directed movements (Tumbar et al., 1999). Finally, the extent of movement of individual tagged chromosomal domains varies depending upon their nuclear environment; specifically, chromosomal domains located at the nuclear periphery or chromocenters move in a smaller diameter than those regions located in the nuclear interior. Taken together, such studies demonstrate the utility of using tagged chromosomal domains in living cells to study the interplay of chromosome dynamics and gene regulation in space and time.

1.7.2: Forced Repositioning of Chromosomal Domains Using Molecular Tethers in Living Cells: Insights into Consequences of Repositioning

More recently, the *lacO* system has been used to position integrated test genes to the inner nuclear membrane and to test the consequences of such repositioning on gene activity [Figure 1.3; (Finlan et al., 2008; Kumaran & Spector, 2008; Reddy & Singh, 2008)]. Three studies took advantage of targeting strategies using *lacO* sites positioned either well upstream or downstream of a test gene to avoid inhibition of transcription due to binding of LacI fusion proteins (Finlan et al., 2008; Kumaran & Spector, 2008; Reddy & Singh, 2008). These target constructs (containing *lacO* repeats) were then tethered to

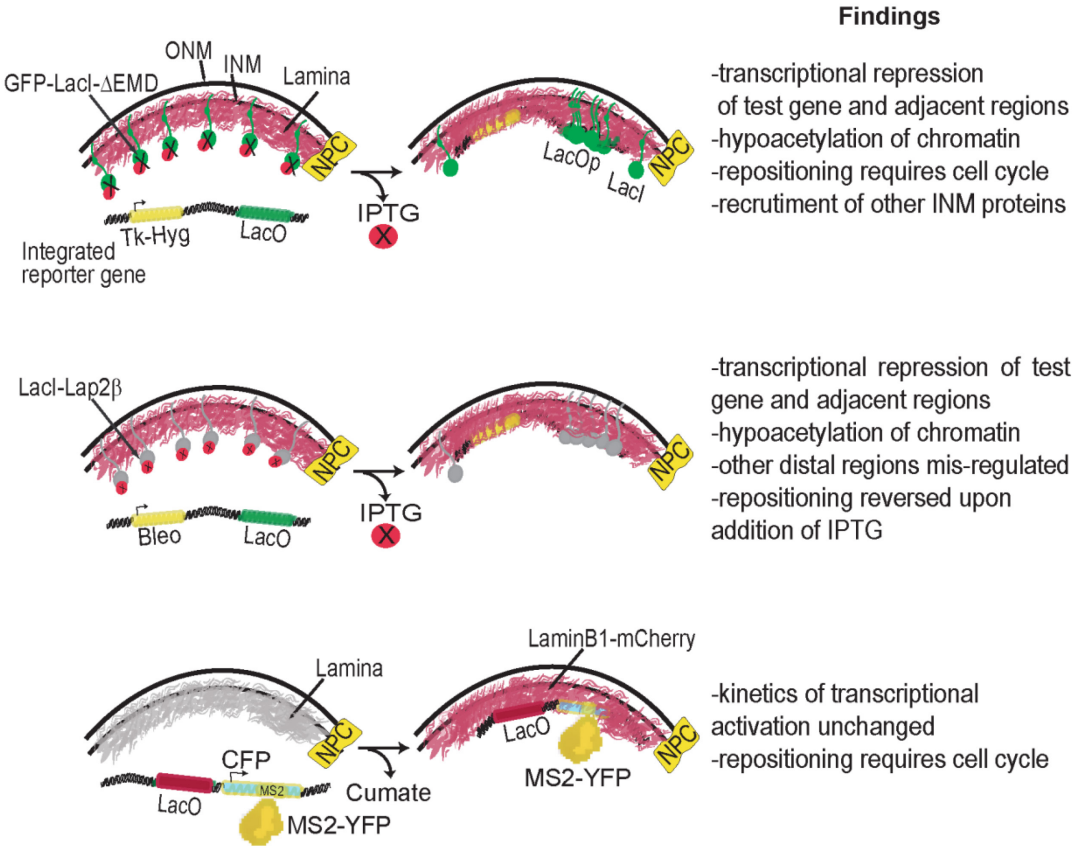


Figure 1.3: Three different strategies expand upon the original *lacO*/LacI system to tether chromatin domains to the INM/Lamina. The top two panels demonstrate similar strategies using an INM protein. In the topmost panel, a truncated version of Emerin, delta Emerin, fused to LacI was used to reversibly interact with chromosomal domains (Reddy et al., 2008). In the middle panel, the INM protein L2b was used as the molecular tether to recruit *lacO* sites (Finlan et al., 2008). In both cases binding was able to be controlled by introducing the allosteric inhibitor IPTG. In the bottom panel, a cumate inducible LaminB1-LacI fusion was used to tether *lacO* insertions to the nuclear lamins (Kumaran and Spector, 2008). The findings from each of these studies are outlined to the right.

the INM/lamina using either a portion of the resident INM proteins (Emerin or Lap2β) or the nuclear lamina protein Lamin B1. In both studies using INM protein domains, the chimeric proteins were stably expressed in cells and they resulted in the recruitment of the target *lacO* containing chromosomal domains to the INM upon removal of IPTG (Finlan et al., 2008; Kumaran & Spector, 2008; Reddy et al., 2008). In the case of the Lamin B1-LacI chimeric fusion, the protein itself was inducibly expressed and was not regulated by IPTG. In all cases, the *lacO* insertions were reproducibly recruited to the nuclear periphery, allowing, for the first time, a study of the consequences of forced compartmentalization. The two studies utilizing INM proteins (Emerin and Lap2β) as tethers resulted in transcriptional repression accompanied by changes in chromatin status around the recruited domain (Finlan et al., 2008; Kumaran & Spector, 2008; Reddy et al., 2008). Intriguingly, the study using Lamin B1 focused on the kinetics of gene induction of a reporter gene tethered to the nuclear lamina (Kumaran & Spector, 2008). The activity of this reporter gene, encoding the cyan fluorescent protein (CFP) and containing MS2-RNA repeats in the intron of the nascent RNA which can be recognized by MS2-YFP protein, allows for visualization of gene activity in individual live nuclei. For this reporter system activity levels were measured either by CFP fluorescence or MS2-YFP accumulation at sites of nascent transcription (Kumaran & Spector, 2008). These forced tethering studies also uncovered a potential link between progression through the cell cycle and recruitment to the nuclear lamina. These observations would never have been made using molecular or fixed cell approaches. These adaptations of the original LacI/*lacO* system demonstrate the versatility of using live cell systems to discover not only dynamics of nuclear architecture but also consequences of nonrandom association with a nuclear compartment.

1.7.3: The Future: Induced Repositioning of Chromosomal Domains Using 'Nuclear Zipcodes' or Specific trans-Factors Identified by Genome-Wide/Molecular Analyses

How is the architecture of a nucleus set up? This is one of the big questions in the field of nuclear dynamics. The studies detailed above describe artificial systems where genic regions are 'forced' to associate with a nuclear domain by molecular tethers, specifically the INM/nuclear lamina and, thus, did not address this basic question. In addition, Dam-ID studies have indicated sequences that are in high-frequency association with this domain (Guelen et al., 2008; Peric-Hupkes et al., 2010). Thus, the preferential association of a genomic region with this structure affords the unique opportunity to uncover the role that specific DNA elements have in determining genome organization. By definition, such cis-elements or nuclear 'zipcodes', would direct or facilitate association of chromosomal domains with a specific nuclear compartment. Using data generated by genome-wide approaches, potential DNA elements can be tested for their ability to direct nuclear compartmentalization (see Chapter 2, Harr et al., 2015; Zullo et al., 2012). The advantage of using such a system is that the elements tested would be subject to the normal cell cycle and other cellular controls involved in establishing nuclear architecture – a feature missing from the forced association studies using molecular tethers described above. One can imagine two ways to test the ability of cis-elements to reposition an ectopic site to a new position in the nuclear volume in living cells. First, one could simply randomly integrate these elements with *lacO* sites and assay the frequency of association with a given compartment (e.g., the nuclear lamina) by looking at GFP-LacI foci by light microscopy. Unfortunately, such an approach yields multiple integrations and different sites for each different sequence tested, making any conclusions about repositioning suspect. Moreover, since there is no control over where the insertions take place, analyzing that the consequences of repositioning would not be possible.

A better strategy would employ a system that allowed for directed insertion of the cis sequences to be tested into the same chromosomal location. An example of a modification of such a system that has been demonstrated to work in this way is shown in Figure 1.4. This tagged chromosomal insertion site (TCIS) system incorporates the ability to tag a chromosomal region via the lacO/LacI system and to recombine specific sequences into the same location using recombination-mediated cassette exchange (RMCE), a well-documented method for site-specific integration of DNA sequences [see chapter 3 for a more detailed description and methodology, (Belmont et al., 1999; Feng et al., 1999)]. The TCIS system represents a rapid way to assay DNA elements for nuclear disposition and activity. Moreover, since multiple sequences are tested within the same nuclear environment, data artifacts due to position effects are avoided.

In addition to using live cells to study the mechanism by which cis- or trans-elements (protein interactors) are able or sufficient to cause a chromosomal domain to reposition to a new nuclear domain, live cells can also be used to detect the molecular association of engineered chromosomes with a protein present in a given nuclear compartment. BiFC allows the direct visualization of protein complexes in their native environment. BiFC is based on the formation of a fluorescent protein from two nonfluorescent constituents (Kerppola, 2008). Specifically, BiFC employs a strategy that splits either GFP or YFP into two domains (an N- and a C- terminal portion) that can complement one another to form a fluorescent protein, but individually have no fluorescence. The two domains only fluoresce when they are brought into close molecular proximity to one another, for example, when they are fused to two proteins that interact with one another. Coupling this technology with the *lacO*/LacI system should enable detection of such nuclear events as chromosome looping and compartmentalization by fusing one-half of the BiFC to LacI and the other half to either a chromatin interacting protein or a protein resident in a specific nuclear compartment, for

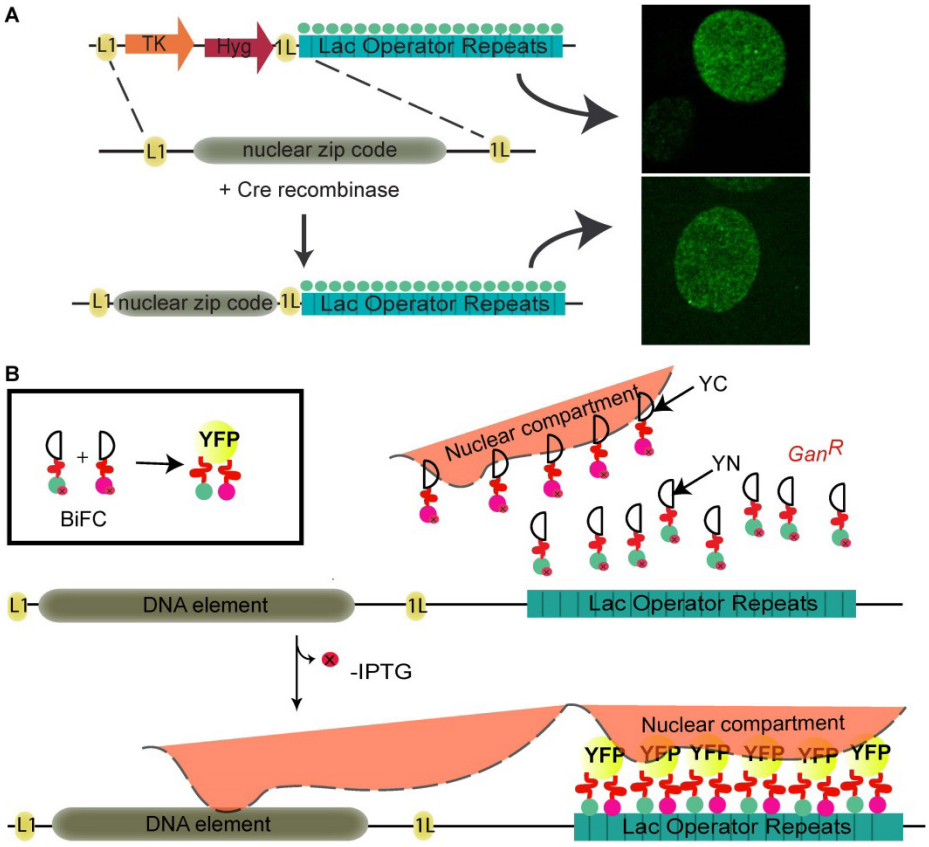


Figure 1.4: Newer uses of the *lacO*/LacI system. (A) A new system has been designed to allow for integration of 'nuclear zipcodes into a specific genomic site. This is accomplished by flipping the sequence of interest into an engineered chromosome region containing inverted loxP sites adjacent to *lacO* sequences. In the absence of a sequence that is able to direct compartmentalization, the array is located in the nuclear interior (nucleus at top). After introducing a targeting sequence containing a putative nuclear zip code, the array is now preferentially located at the nuclear periphery (nucleus at the bottom). Analysis using BiFC (see below) or expression from a test gene can be incorporated into this system to garner positional, molecular and regulatory information. (B) Design of a system to detect molecular interaction of a genomic region with a nuclear compartment. The basic concept of BiFC is illustrated in the box in panel (B). LacI is fused to one-half of fluorescent YFP and a protein located in a nuclear subcompartment is fused to a cognate half of the YFP molecular. If the two proteins are in molecular proximity, a complete fluorescent YFP will be formed. A nuclear zipcode is used (as is (A)) to direct a sequence to a subnuclear domain. The LacI fusion protein bound to *lacO* repeats will be directed to the compartment and will be brought into proximity to allow for formation of the fluorescent protein. The type of analysis allows one to get specific real time information about molecular interactions.

example, the nuclear lamina (Figure 1.4 and see Appendix I). BiFC has been used quite successfully to demonstrate novel dynamics of the protein CBX binding to chromatin (Vincenz & Kerppola, 2008). CBX recruits the polycomb repressive complex 1 (PRC1) and is very important in establishing heterochromatin domains. Numerous previous studies relied on immunofluorescence to detect CBX proteins; however, such a strategy is unable to detect whether the protein is actually interacting with chromatin. Of course, ChIP can detect specific chromatin interactions, but those studies lack spatial information. Using BiFC to CBX and histones, the patterns of chromatin interacting versus total CBX were quite distinct (Vincenz & Kerppola, 2008). Thus, BiFC allows measurement of spatial, contextual, and temporal changes in protein complexes and will likely be an important tool in detecting the dynamics of nuclear compartmentalization.

Live cell imaging techniques are poised to take advantage of the many genome-wide molecular assays that are currently being employed to describe genome-wide nuclear associations to uncover the mechanisms and consequences of nuclear architecture and organization on gene activity. Using the well-established *lacO*/LacI system and other techniques such as BiFC and nascent RNA detection methods in living cells will enable spatial, temporal, and molecular analysis to be carried out in a single cell giving an unprecedented view of form and function of nuclear dynamics.

1.8: Summary

The work described in subsequent chapters demonstrates the use of genome wide data, generated by Dam-ID, to identify specific regions of the genome that are variably associated with the nuclear periphery (variably lamina associated domains, vLADs) that are enriched for developmentally regulated genes. We propose that these vLADs play a key role in regulating cell type specific genes that are involved in development. To understand the mechanism behind vLAD establishment and

maintenance at the nuclear periphery, we utilized the TCIS system in murine fibroblasts to determine DNA sequences sufficient to target to the nuclear periphery and therefore reorganize chromatin. Through this investigation, which is presented in Chapter 2, we further identify proteins involved in this process, including Ying-Yang-1 (YY1), BTB/POZ domain binding proteins and polycomb repressive complex 2 (PRC2) and the importance of at least two histone modifications, H3K27me3 and H3K9me2/3, in mediating this process. We describe the development of a novel tool for studying both DNA elements and their protein partners involved in nuclear compartmentalization, the Tagged Chromosomal Insert Site (TCIS) System, in detail in Chapter 3.

Chapter 2

Directed targeting of chromatin to the nuclear lamina is mediated by chromatin state and A-type lamins

This work was done in collaboration with Teresa Luperchio, Xianrong Wong, Erez Cohen, Sarah J. Wheelan, and Karen L. Reddy. It was published in *The Journal of Cell Biology*, January, 5th 2015

2.1: Abstract

Nuclear organization has been implicated in regulating gene activity. Recently, large developmentally regulated regions of the genome dynamically associated with the nuclear lamina have been identified. However, little is known about how these lamina-associated domains (LADs) are directed to the nuclear lamina. We use our tagged chromosomal insertion site system to identify small sequences from borders of fibroblast-specific variable LADs that are sufficient to target these ectopic sites to the nuclear periphery. We identify YY1 (Ying-Yang1) binding sites as enriched in relocating sequences. Knockdown of YY1 or lamin A/C, but not lamin A, led to a loss of lamina association. In addition, targeted recruitment of YY1 proteins facilitated ectopic LAD formation dependent on histone H3 lysine 27 trimethylation and histone H3 lysine di- and trimethylation. Our results also reveal that endogenous loci appear to be dependent on lamin A/C, YY1, H3K27me3, and H3K9me2/3 for maintenance of lamina-proximal positioning.

2.2: Introduction

Recent evidence suggests that nuclear architecture influences gene regulation through establishment of large chromatin domains and through enrichment of regulatory and structural proteins within these regions (Misteli, 2005; Scaffidi and Misteli, 2006; Cremer et al., 2001, 2006; Fedorova and Zink, 2008; Elcock and Bridger, 2010; Ferrai et al., 2010; Van Bortle and Corces, 2012). One such domain, the nuclear periphery, is comprised of the inner nuclear membrane, resident inner nuclear membrane proteins, as well as underlying nuclear lamina and associated proteins. This region has been implicated in gene regulation, and various studies demonstrate that recruitment of genic regions (lamina-associated sequences [LASs]) to the nuclear periphery is sufficient to cause repression and silencing of associated genes (Finlan et al., 2008; Reddy et al.,

2008; Zullo et al., 2012). More recently, molecular mapping of large chromatin regions in molecular contact with the nuclear periphery by DNA adenine methyltransferase (Dam) identification (DamID) has identified large lamina-associated domains (LADs; 0.1–10 Mb) that dynamically associate with the nuclear lamina (Guelen et al., 2008; Peric-Hupkes et al., 2010). Moreover, cell state–specific association with the nuclear lamina appears to be involved in repression of many developmental genes, including the immunoglobulin heavy chain (*Igh*) locus (Kosak et al., 2002; Yang et al., 2005; Williams et al., 2006; Szczerbal et al., 2009; Meister et al., 2010; Peric-Hupkes et al., 2010; Yao et al., 2011; Lin et al., 2012). More recently, both LMNA (lamin A) and lamin B receptor have been implicated in tethering heterochromatin to the nuclear lamina in rod photoreceptor cells in nocturnal animals (Solovei et al., 2013). LADs have been reported to be repressive domains enriched in histone H3 lysine 9 di- or trimethylation (H3K9me2/3; Guelen et al., 2008; Wen et al., 2009; Towbin et al., 2012). In addition, two recent studies have implicated H3K9me2/3 in the positioning of chromatin at the nuclear lamina in murine cells and in *Caenorhabditis elegans* (Towbin et al., 2012; Bian et al., 2013). Intriguingly, the borders of LADs appear to be enriched in both H3K9me2/3 and H3K27me3 (histone H3 lysine 27 trimethylation) as well as CCCTC-binding factor (CTCF) binding sites; however, a role for the chromatin state found enriched in these regions in establishment and/or maintenance of LAD organization has not been thoroughly investigated (Guelen et al., 2008; Zullo et al., 2012; Meuleman et al., 2013; Van Bortle et al., 2013). It is of special note that these LAD borders, enriched in H3K27me3 and flanked by CTCF binding sites, are quite sharp and well delimited, suggesting an active mechanism to continually reestablish and maintain these regions. Because many of the developmentally regulated variable LADs (vLADs) between cell types occur by shifting these LAD border regions, we hypothesized that the study of border regions of vLADS would enable a greater understanding of how the dynamic

genome is reorganized at the nuclear periphery (Figure 2.1; Peric-Hupkes et al., 2010). We therefore sought to elucidate factors and genic elements involved in positioning of chromatin to the nuclear periphery in mammalian cells, with a particular focus on dynamically reorganized border regions of LADs. This work identifies genomic regions containing developmentally regulated genes that reside in regions that are dynamically lamina associated depending on cellular state (vLADs) and, therefore, have regulated nuclear positioning. We have identified vLADs covering the Ikaros (*Ikzf1*) and B cell CLL/lymphoma 11A (*Bcl11a*) loci, which are positioned at the border of fibroblast (FB)-specific vLADs and are no longer associated with the lamina in pro–B cells. We have developed and used our tagged chromosomal insertion site (TCIS) system to examine single DNA sequences from these vLAD border regions for their ability to target and scaffold chromatin to the nuclear periphery. This approach has led to the discovery of a role for LMNA/C, YY1, H3K27me3, and H3K9me2/3 for targeting to the nuclear lamina of both ectopic and endogenous LADs.

2.3: Results

2.3.1: Developmental and cell type–specific genes are enriched in vLADS

The *Igh* locus, which itself comprises a vLAD, is lamina proximal and inactive in FB but is centrally disposed and active in pro-B cells where it is transcriptionally and recombinationally active (Figure 2.1 A; Reddy et al., 2008). We hypothesized that there would be other vLADs between FB and pro–B cells that contain developmentally regulated genes. To determine whether such regions exist, we detected *in vivo* lamina–genome interactions by performing DamID in pro–B and FB cells (see Materials and methods; Vogel et al., 2007; Reddy et al., 2008). We determined LADs to be contiguous (>50 kb) regions exhibiting higher signal from Dam-LMNB1 (lamin B1) relative to the

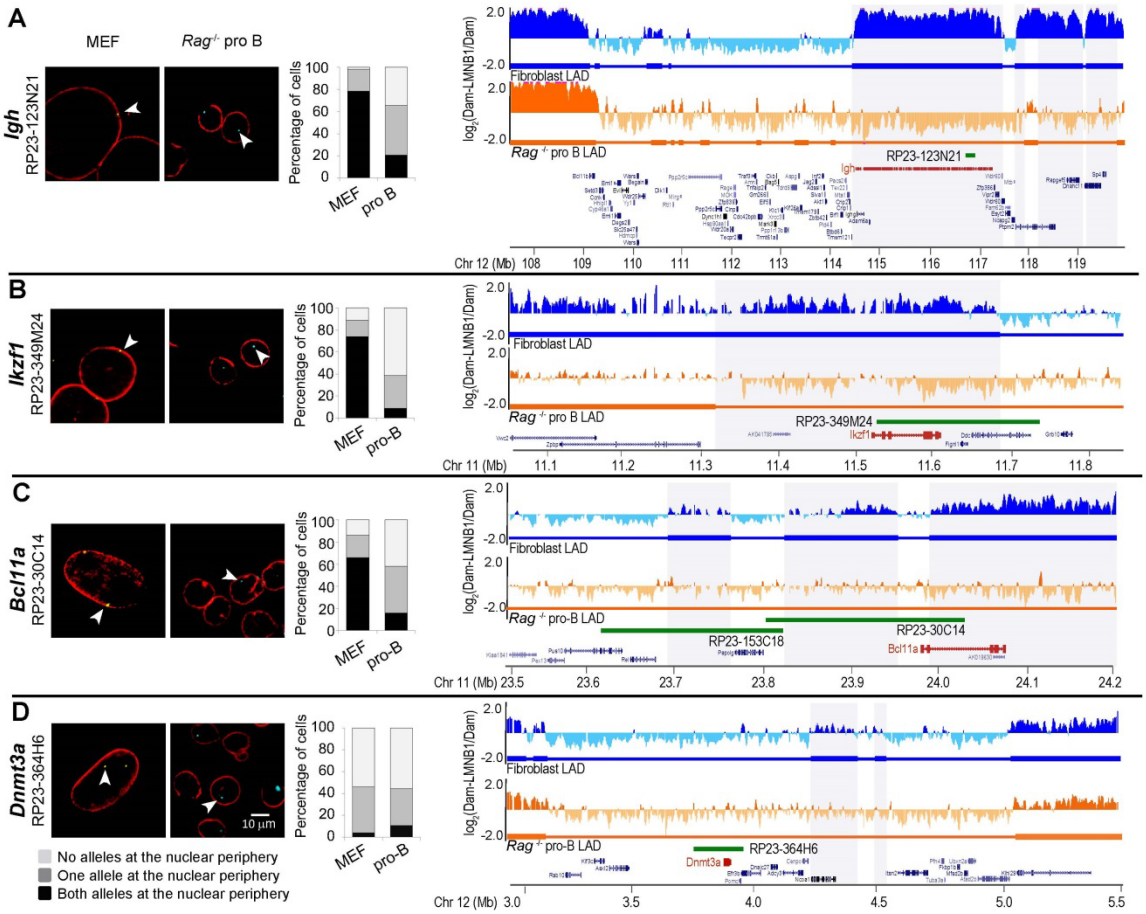
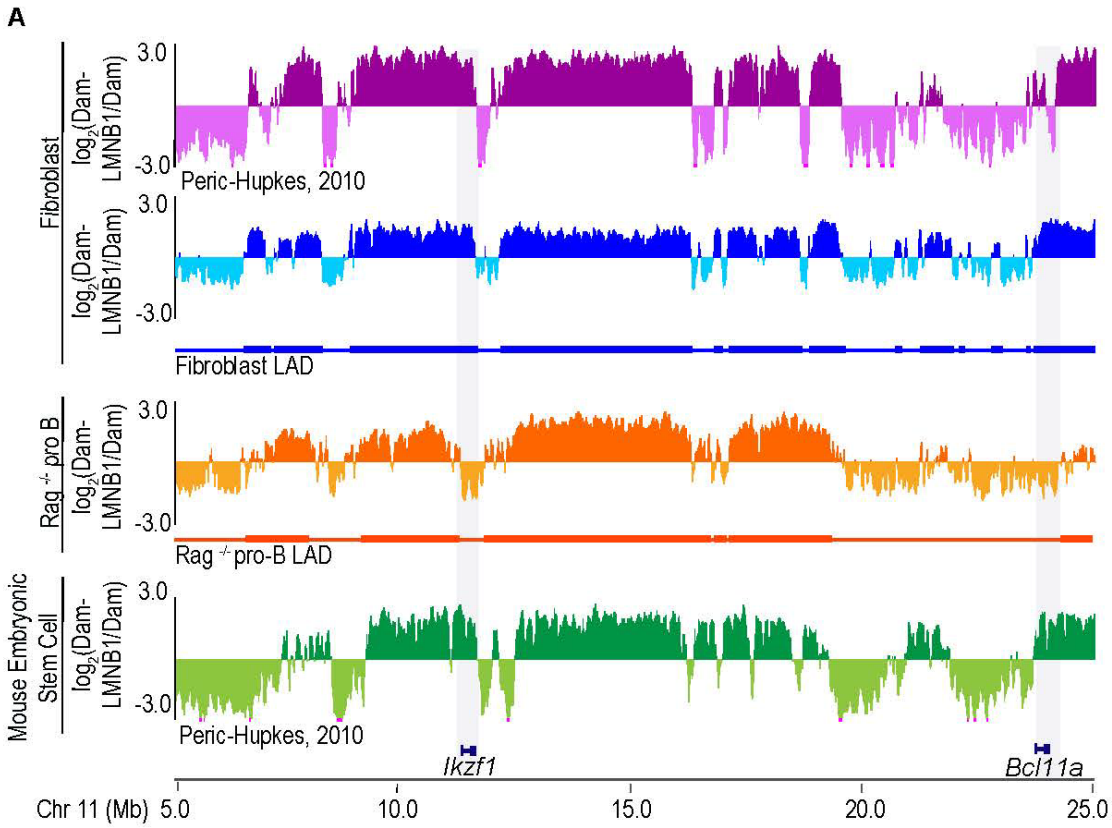


Figure 2.1: Both FB and proB cell genomes contain vLADs. (A-D) Shown are regions containing the *Igh, Ikzf1* and *Bcl11a* loci from FB specific vLADs. Left, representative images of 3D DNA ImmunoFISH of endogenous in FB and proB cells. FISH probes detecting the indicated regions (arrowhead) and the nuclear lamina is demarcated by LMNB1 (red). Middle, quantitation of peripheral association was determined by overlap of FISH probe and LMNB1 (n ≥ 50). Right, DamID detection of LADs log_2(Dam-LMNB1/Dam) average ratios from three experiments for FB (blue) and proB cells (orange). Traces above the zero line indicate a region in a LAD. Solid blue and orange bars underscore LADs in FB and proB cells, respectively. Genes are indicated as transcripts (blue) our gene of interest is red, BAC locations are indicated by green bars and gray shading indicates a FB specific vLAD.

background control, Dam only (log_2 [Dam-LMNB1/Dam]), in mouse embryonic FBs (MEFs) and proB cells (Figure 2.1, solid heavy blue or orange lines under histograms denote identified LADs; see Materials and methods; Venkatraman and Olshen, 2007; Zullo et al., 2012). These data are in agreement with previously published LAD data for MEFs (Figure 2.2 A; Peric- Hupkes et al., 2010). In agreement with a previous study, we identified numerous vLADs between FB and proB cells that contain key developmental and cell type–specific genes (Figure 2.1; Peric-Hupkes et al., 2010). For this work, we focused on genes that were resident in LADs in FB but were not lamina-proximal in pro–B cells.

Not surprisingly, we demonstrate that the *Igh* locus is contained within one of these vLADs (Figure 2.1 A; Reddy et al., 2008; Zullo et al., 2012). In addition, we have also determined that the key B cell development genes *Ikzf1* and *Bcl11a* are contained within FB-specific vLADs (but not in proB cells) as determined both by immuno-FISH and DamID protocols, consistent with their (active) roles in B cell development (Figure 2.1, B and C; and Figure 2.2 A). Therefore, these regions are ideal candidates for interrogating sequence-dependent mechanisms of positioning to the nuclear lamina. We also identified a region spanning the *Dnmt3a* gene, which is not in a LAD, but inactive (by array and quantitative PCR [qPCR] analyses), in our cell lines (Figure 2.1 D). 3D immuno-FISH analyses confirmed that the disposition of these endogenous genomic regions in MEFs and proB cells are as expected (Figure 2.1). It is important to note that the loci/regions we have identified in MEFs as lamina proximal by DamID are at the nuclear periphery in a majority of FB (65–80% have both alleles at the lamina), whereas these same regions display negative lamina association by DamID and an association rate of 30% in proB cells, reflecting the background level of “association” with the lamina by cytological measures. Both of these cell types are primary cells and may display different levels of association with the lamina when compared with data



B

Clone Name	Gene
RP23-123N21	*Igh*
RP23-349M24	*Ikzf1*
RP23-30C14	*Bcl11a*
RP23-153C18	*Bcl11a*
RP23-364H6	*Dnmt3a*

Figure 2.2: Lamin associated domains, as determined by DamID compared in three cell types. (A) A 20kb window shows that FB Dam-ID traces (blue) align with previously published data from this region (purple, Peric-Hupkes, 2010 GEO: GSE17051). DamID data is represented by $\log_2$(Dam-LMNB1/Dam) average ratios from three experiments, shown for FB (blue) and proB cells (orange). Traces above the zero line indicate a region in a LAD. Mouse $Rag^{-/-}$ proB cells (orange) and Mouse Embryonic Stem cells (green, Peric-Hupkes, 2010 GEO: GSE17051) highlight the presence of *Ikzf1* and *Bcl11a* in FB specific vLADs (vLADs shaded in gray). Solid blue and orange bars are bioinformatically determined binary LAD calls for FB and proB cells, respectively. Genes are indicated as transcripts below the traces (blue). (B) BACs to the LAD border regions utilized in 3D DNA ImmunoFISH and nuclear repositioning assays.

published from subcloned cancer-derived cell lines (Kind et al., 2013). Finally, gene expression microarray data from proB cells and FB demonstrate differential expression levels for *Bcl11a, Ikzf1*, and *Igh*, in agreement with previous studies and consistent with the nuclear lamina facilitating or reflecting a transcriptionally repressed state (unpublished data; Medina and Singh, 2005; Johnson et al., 2008, 2009; Reynaud et al., 2008; Heydarian et al., 2014).

Interestingly, we have noted that many vLADs are at LAD borders, suggesting that these regions may be especially important in dynamic genome reorganization (Peric-Hupkes et al., 2010 and unpublished data). The *Igh* locus is unusual in that the locus itself comprises the LAD (3 Mb) and the entire LAD reorganizes in proB cells, whereas *Ikzf1* and *Bcl11a* are more typical examples of vLADs, with only a portion of the LAD (proximal to the border) being lost in the permissive proB cell type (Figure 2.1).

2.3.2: DNA sequences from vLAD borders target to the nuclear periphery

To elucidate the ability of specific LASs to direct targeting of sequences to the nuclear periphery, we integrated bacterial artificial chromosome (BAC) DNA constructs carrying sequences from FB-specific vLAD border regions covering the *Ikzf1* (RP23-349M24) and *Bcl11a* (RP23-30C14 and RP23-153C18) loci into the genome of NIH3T3-derived C57BL/6 FB (ATCC CRL-2752; Figures. 2.2 B and 2.3, A and B). As controls, a BAC from the distal, but internal, portion of the *Igh* locus (lamina-associated positive control, RP23-123N21) and another covering the *Dnmt3a* locus (non-lamina-associated negative control, RP23-364H6) were also used (Figures 2.1 and 2.3; Zullo et al., 2012). These BACs were randomly co-integrated into FB cells with hygromycin-selectable *lacO* arrays, as previously described (Zullo et al., 2012). The *lacO* arrays serve as docking sites for EGFP-LacI, thus enabling quick identification by microscopy of the disposition of the integrations. An overlap of LMNB1 signal and an EGFP-LacI/ *lacO* focus was scored

Figure 2.3: Bacterial Artificial Chromosomes (BACs) covering developmentally regulated vLAD borders target to the nuclear periphery. (A) Representative images of random co-integration of BACs and *lac*O arrays. Arrowheads indicate the *lac*O array location as detected by EGFP-LacI binding. LMNB1 (red) marks the periphery of the nucleus. Inset is a 300X magnification of the EGFP-LacI focus (B) Quantitation of peripheral association was determined by overlap of EGFP-LacI focus and anti-LMNB1 signal ($n \geq 50$, $P \leq 0.001$). The dotted line indicates the frequency that a non-targeting sequence associates with the nuclear periphery (*lacO* arrays) Error bars indicate SD of two experiments by two individuals. (C) DNA fragments covering the border region of the FB specific *Bcl11a* vLAD contain LADed and non-LADed regions. Most of these fragments do not cover the *Bcl11a* gene. Histogram traces are $\log_2$(Dam-LMNB1/Dam) and consecutive positive signal indicates a LAD region in FB. Solid blue bars represent binary LADs calls. The *Bcl11a* locus (dark blue) is shown above the LAD call and the position of the RP23-153C18 and RP23-30C14 BACs used to generate the smaller fragments are shown in green. Smaller DNA fragments tested for repositioning are shown in pink (generated from the RP23-153C18 BAC) and orange (generated from the RP23-30C14 BAC). (D) Quantitation of random integration of the indicated DNA fragments (orange) from BAC RP23-30C14 in C57BL/6 FB ($n \geq 50$, $p \leq 0.001$). Error bars are the D of imaging data as scored by two individuals. (E) Quantitation of IF data from TCIS clones Y (black) and 12 (gray) after switching the indicated DNA fragments (pink) contained within BAC RP23-153C18. TCIS-NS are the TCIS clones Y and 12 containing the non-recombined or non-switched TCIS system.

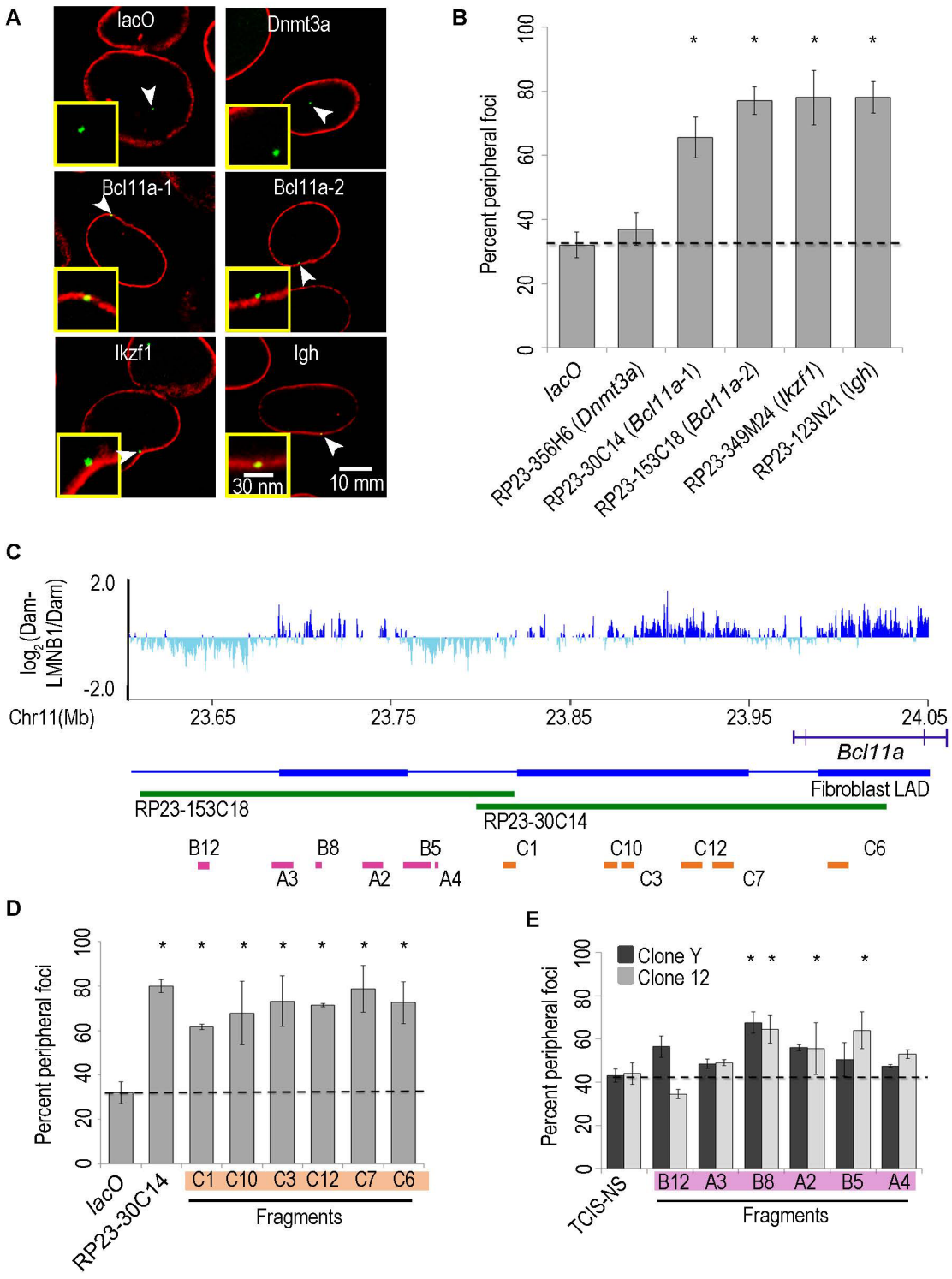


Figure 2.3: Bacterial Artificial Chromosomes (BACs) covering developmentally regulated vLAD borders target to the nuclear periphery.

as peripheral (Figure 2.3, A and B). *lacO* arrays integrated alone served as a control for expected distribution of random integrations, and these were found at the periphery with a frequency of 30% (Figure 2.3 B). Importantly, an integrated BAC covering the *Dnmt3a* locus is centrally disposed despite its inactive status, reminiscent of the disposition of the endogenous locus (Figures 2.3 B and 2.1 D). We note that a previous study showed that the genomic region around the *neuronal PAS domain 3 gene* (*NPAS3*) was not in a LAD in FBs (though inactive) and was unable to reposition an ectopic site in this cell type, in agreement with our results for the genomic region around *Dnmt3a* (Zullo et al., 2012). Moreover, *NPAS3* is in a LAD in proB cells (unpublished data; GEO accession no. GSE56990). These data suggest that, perhaps, sequences from domains that are not in an FB LAD are unable to confer lamina-proximal association in FB, regardless of transcription status or LAD status in alternate cell types. In contrast, BACs covering vLADs have a 60% (*Bcl11a* - 1) to 80% (*Ikzf1*, *Bcl11a*-2, and *Igh*) propensity to be lamina proximal, mimicking the disposition of endogenous loci (Figs. 2.1 and 2.3 B).

Given that these BAC LAS are able to mediate association of ectopic sites to the nuclear lamina, we next asked whether we could elucidate smaller sequences capable of such targeting. Both the *Ikzf1*- and *Bcl11a* -containing BACs demonstrating potential to direct to the nuclear periphery were further fragmented (*Ikzf1* (A–O); Figures 2.4 A and 2.3 C), and these smaller potential *Ikzf1* LASs were randomly co-integrated with the aforementioned *lacO* arrays. The *Ikzf1* fragments tested ranged from 900 bp to 30 kbp and were derived from regions interior to the vLAD border as well as those crossing and outside of the border regions (Figure 2.4, A–C , *Ikzf1* (I) and (O)). Interestingly, the fragments seem to show repositioning potential that correlated with the LAD status of the endogenous region (Figure 2.4, B and C, gray bars). Noteworthy is the non-relocating *Ikzf1* (F) whose sequence falls in a break, or dip, in lamina association in an otherwise contiguous LAD region (Figure 2.4, B and C, gray bars). Sequence from *Ikzf1* (O) falls

Figure 2.4: *Ikzf1* LAS target to the nuclear periphery in FB. (A) *Ikzf1 (A-I, O)* fragments span the border region of the FB specific *Ikzf1* vLAD. Histograms are $\log_2$(Dam-LMNB1/Dam) and the blue bar underscores the LAD. The *Ikzf1* locus (blue) and overlapping BAC used to generate the smaller fragments are shown (green). *Ikzf1* LAS are red, and non-associating fragments are gray. (B) Representative images of the showing the disposition of *lacO* arrays (arrowheads, green) and LMNB1 (red) in the original TCIS clone as well as nuclei harboring randomly integrated *Ikzf1* BAC fragments (top panels) or TCIS clones with site-specific recombination of *Ikzf1 (A, F, I, O)* Inset, 300X (C) Quantitation of peripheral association was determined by overlap of EGFP-LacI foci and LMNB1 ($n \geq 50$). Fragments were tested for their ability to target to the nuclear periphery by random integration (gray) or by directed integration (TCIS, black bars). Error bars indicate SD. $p \leq 0.001$ (ł, random integration and *, TCIS switch). (D) Representative images of IF for the disposition of *Ikzf1 (I) or Igh (N)* LAS recombined into the TCIS sites in clones Y and 12. Arrowheads, EGFP-LacI binding *lacO* arrays (green) at the TCIS site, LMNB1 (red). Inset, 300X (E) Quantitation of peripheral association of *Ikzf1 (I) or Igh (N)* LAS in clones Y and 12 ($n \geq 50$, $p \leq 0.05$).

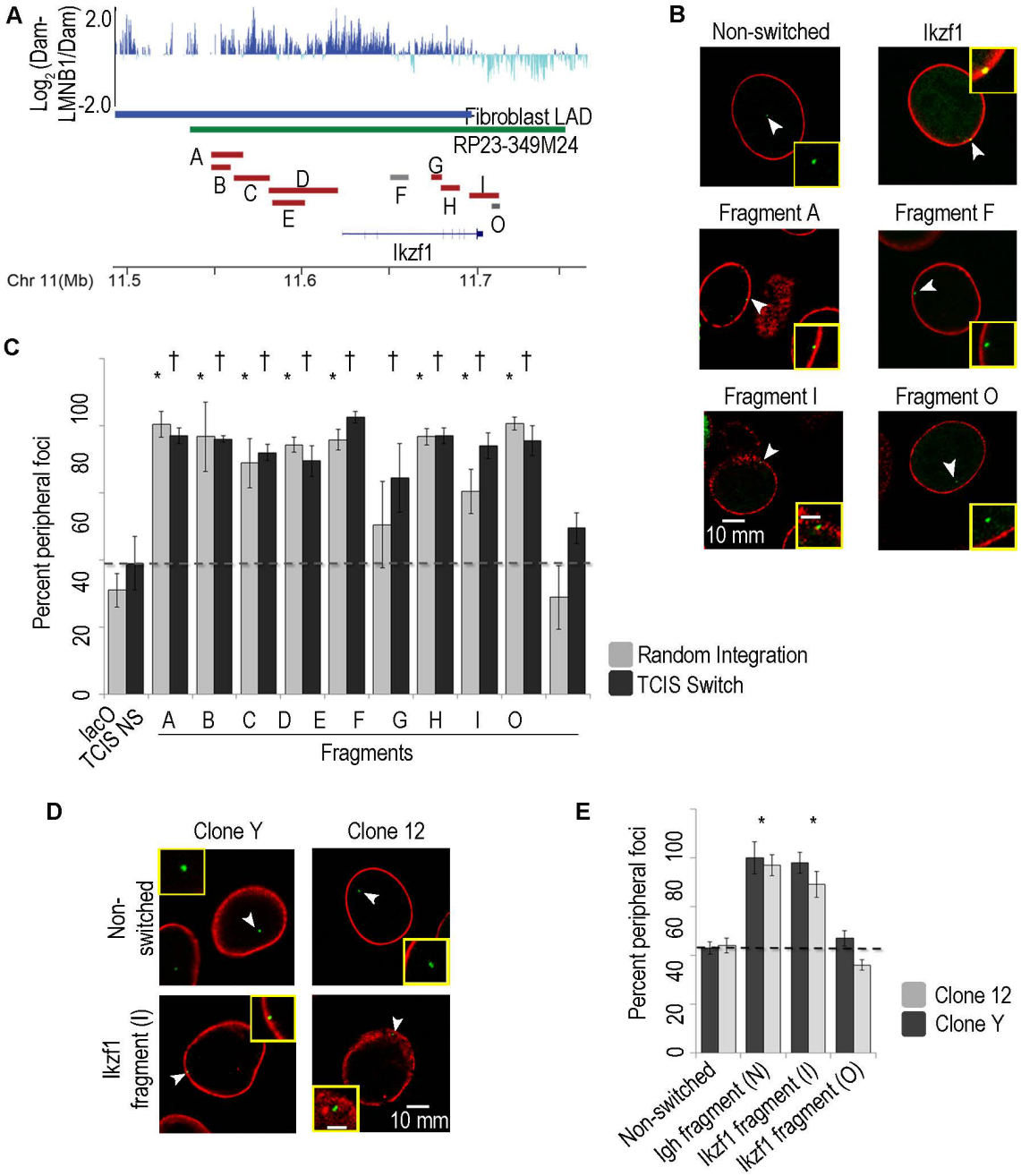


Figure 2.4: Ikzf1 LAS target to the nuclear periphery in FB.

outside of a LAD, and it does not relocate to the periphery (Figure 2.4, A–C, gray bars). We therefore hypothesize that *Ikzf1* (O) lacks sequences sufficient to target (address) to the nuclear periphery, whereas the *Ikzf1* (I) LAS retains this information. Similar results were obtained for the BAC containing sequences from the LAD border covering the *Bcl11a* locus (Figure 2.3 D).

One caveat to this, and previously published experiments, is that the LASs tested have all been randomly integrated and likely represent many copies of the sequence at any given insertion site (Zullo et al., 2012; Bian et al., 2013). Therefore, it is difficult to determine whether an identified LAS was truly sufficient for targeting to the periphery or whether multiple copies of the LAS conferred a novel DNA or chromatin state that was it-self a signal for compartmentalization. Intriguingly, many LADs contain duplicated genes or loci, for example, the *Igh* and *Cyp3a* loci, and are enriched in long interspersed nuclear elements, suggesting that multimeric sequences may be a signal for directing to the lamina (Zullo et al., 2012). However, there are regions of the genome that do not appear to be highly duplicated that are in a LAD (e.g., *Ikzf1* and *Bcl11a* LAD regions), and we therefore hypothesize that a single LAS may contain sufficient information to direct lamina-proximal targeting.

2.3.3: LAS from vLAD borders are sufficient to target to the periphery

To enable an experimental test of this hypothesis, we have developed a novel technique, TCIS, in which we can integrate a single DNA fragment of choice into the genome by directed recombination (Figure 2.5 A). FB clone Y and 12 were identified as carrying a single integration site by qPCR and microscopy (immunofluorescence [IF] or live cell imaging of GFP-enriched foci). These parental clones display the “default” disposition of the single integrated TCIS system, which is away from the nuclear lamina

Figure 2.5: The Tagged Chromosomal Insertion Site (TCIS System) allows for the site specific recombination of a DNA sequence of interest. (A) The TCIS system is composed of two previously described technologies: the *lacO*/LacI system (*lac Operator* / Lac repressor) and Recombination-Mediated Cassette Exchange (RMCE). This system allows us to tag a chromosomal region with *lacO* segments, recruit LacI fusion proteins and to site-specifically integrate specific single copy DNA sequences of interest by Cre-mediated recombination into inverted loxP sites. (Feng, 1999; Robinett, 1996). Specifically, we integrated a linearized construct containing 256 *lacO* repeats contiguous with two inverted loxP sites interrupted by thymidine kinase (TK) and hygromycin (Hyg) genes for negative and positive selection, respectively. TCIS cell lines were generated by introducing this linearized construct into 3T3 derived FB cells followed by positive selection with hygromycin. Individual isolated clones were screened by quantitative PCR (qPCR) for copy number and single sites of integration were verified by fluorescence microscopy of EGFP-LacI, which binds to and accumulates on the integrated *lacO* array, thus marking the insertion site.(B) Quantitation of IF versus FISH shows comparable positional determination of the TCIS system location (n ≥ 50). (C) TCIS insertion sites are centrally disposed and not in a lamina associated domain, as determined by DamID. QPCR to a region of the TCIS locus is not enriched in regions of the genome marked by Dam-LMNB1 when compared to Dam treatment alone. Primers to the *Igh* locus, a lamina associated regions in FB cells, is used as a positive control. Primers to a non-lamina associated regions (dip) are used as a negative control. The data shown are from a single representative experiment, out of three. (D) Efficient and successful 'switching' is tested by recombining in GFP coding gene into the TCIS region. To determine the efficiency by which we can introduce new DNA sequences, we cloned a CMV-EGFP expression cassette into a vector containing inverted loxP sites that corresponded to the loxP sites in the TCIS integration ('switch vector') and FB cells were co-transfected with this construct along with a vector encoding Cre-recombinase into TCIS clone Y or clone 12. Efficient switching takes place within 5d and is guaranteed by negative selection with 1μM gancyclovir for 24h (Feng, 1999). Flow cytometry shows a shift in GFP fluorescence in the whole population (green), compared to non-switched TCIS clones (purple). The data shown are form a single representative experiment out of three.

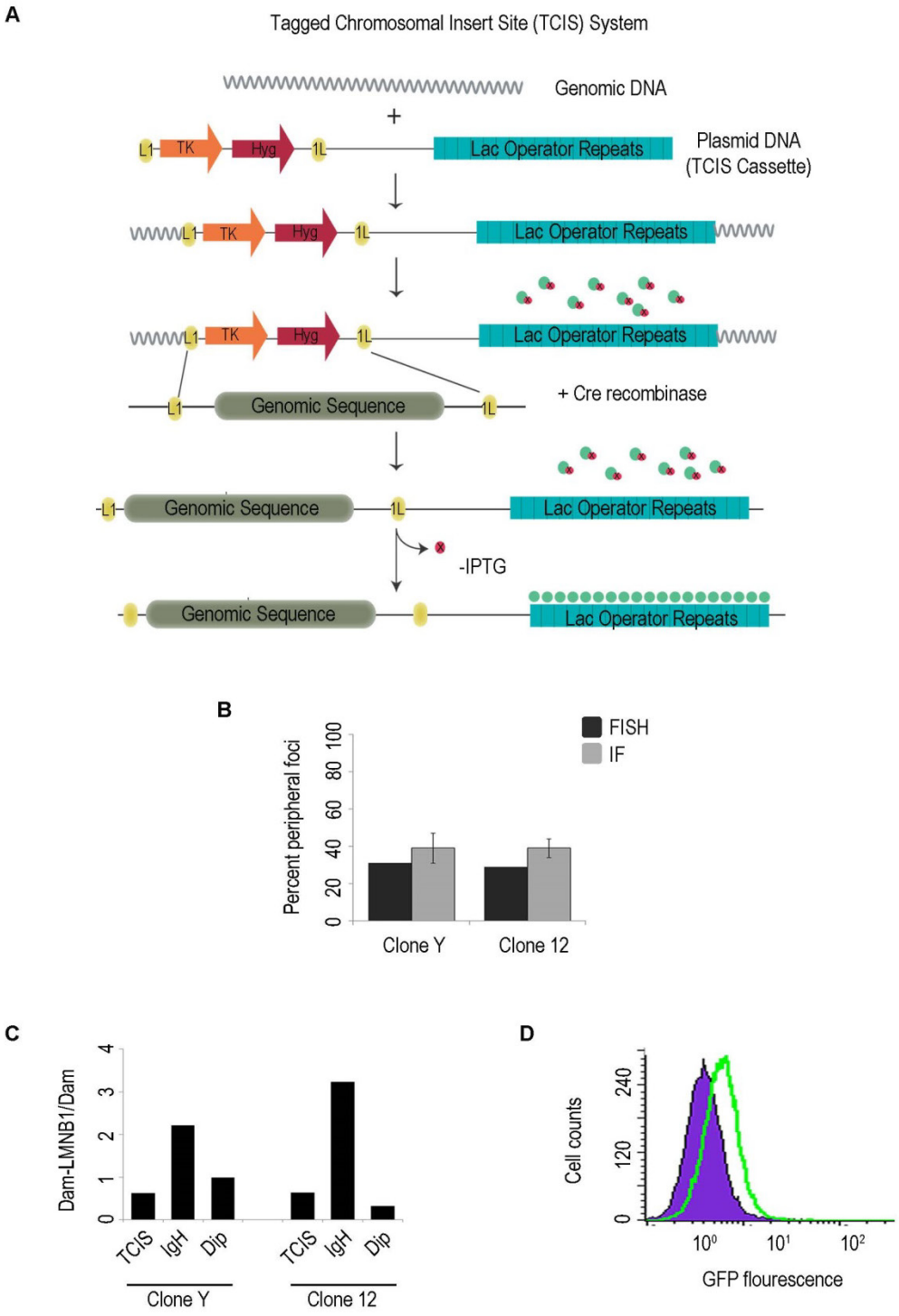


Figure 2.5: The Tagged Chromosomal Insertion Site (TCIS System) allows for the site specific recombination of a DNA sequence of interest.

(Figure 2.4, B and C; and Figure 2.5, B and C). We note that only a subset of the cells display EGFP-LacI foci detectable by 3D microscopy, as previously described (Belmont et al., 1999). Therefore, to ensure that we are able to measure disposition accurately using IF and EGFP-LacI foci as a readout, we demonstrated that 3D immuno-FISH to the lacO sequences reflected the same disposition as our IF strategy (Figure 2.5 B). Although we do not know the integration sites, the TCIS region is not associated with the nuclear lamina by DamID analyses, thus the default association is away from the nuclear lamina (Figure 2.5 C). Using this system, we are able to "switch" DNA segments into the TCIS system with high efficiency in as little as 5 d, with selection (Figure 2.5 D). Thus, the TCIS system enables testing of a single non-iterated LAS for its ability to target an ectopic genomic site to the nuclear periphery. Moreover, each LAS tested will be in the exact same genic location, thus mitigating misinterpretation of results based upon possible position effects caused by differential integration sites.

The previously described *Ikzf1* (A–O) fragments were therefore integrated into TCIS clone Y expressing EGFP-LacI to enable detection of disposition of the *lacO* sites (Figure 2.4, A–C, black bars). These potential LASs in single copy showed relatively the same probability of targeting to the nuclear periphery in FB cells as they did in multiple copy random integrations (Figure 2.6 C). The *Ikzf1* (I) LAS (border) and a control from *Igh* LASs were tested in both of our clones Y and 12 (Figure 2.4, D and E; Zullo et al., 2012); additionally, fragments from broader border regions near *Bcl11a* were integrated into clones 12 and Y, and similar results were obtained (Figure 2.3 E). The transitional *Ikzf1* (I) LAS, which covers the outermost edge of the *Ikzf1* LAD border region, was then further dissected to identify specific DNA elements with roles in establishing and/or defining a LAD. Specifically, 2.5-kbp subfragments covering the entire *Ikzf1* (I) were switched into clone Y. The smaller *Ikzf1* (I) LAS fragments comprised of sequences derived from regions adjacent to borders, but outside of LADs, are unable to mediate

Figure 2.6: YY1 binding sites are enriched in LAS. (A) Profiles of aligned LAD border regions (Chr 9-14; left and mirrored right border regions combined) are shown for Lamin B1 binding, YY1 and CTCF binding site density and H3K27me3. To align LAD borders, genome-wide positions of all analyzed features were converted to coordinates relative to the nearest border. Grey area and positive coordinates, inside LADs; white area and negative coordinates, outside LADs. (B) DamID histograms in FB (blue), ESC cells (green, Peric-Hupkes, 2010, GSE17051) and proB cells, orange. Histograms are $\log_2$(Dam-LMNB1/Dam) and positive signal indicates a LAD region. The solid blue and orange bars underscore LADs. The *Ikzf1* locus (blue) and BAC used to generate the smaller fragments (green), is shown below the fragments. LAS are red and non-repositioning fragments are gray. (C) Depicts magnification of the *Ikzf1* LAD border (light yellow from panel C). Sub-fragments (1-9) of the *Ikzf1(I)* LAS are shown relative to ChIP-Seq data from ESC and proB cells show YY1 binding site enrichment (YY1, GSM628031, proB, GSM100256, Mendenhall, 2010; Verma-Gaur, 2012).

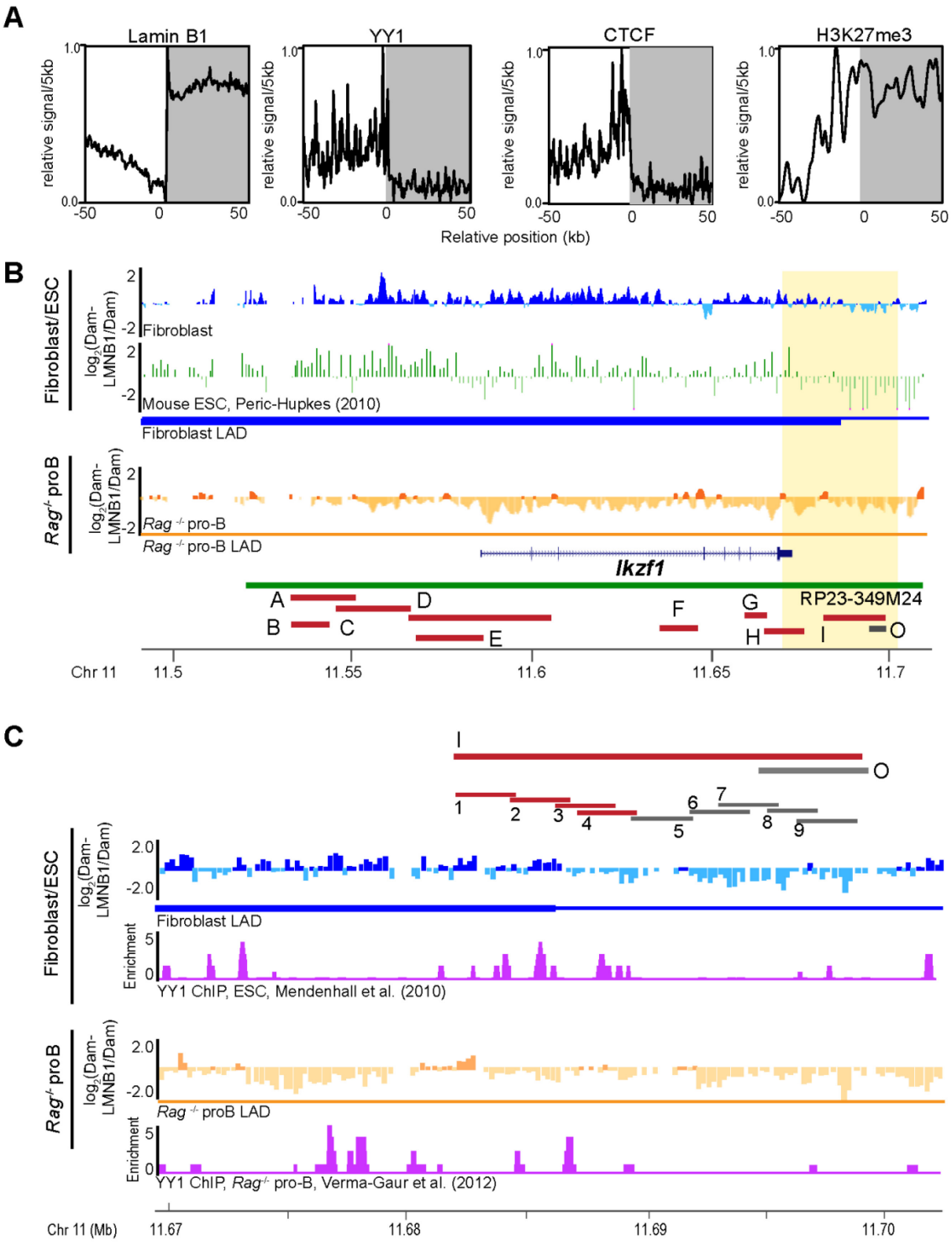


Figure 2.6: YY1 binding sites are enriched in LAS.

repositioning (Figure 2.7). We note that *Ikzf1* (I) LAS fragments 3 and 4 straddle the defined border region. These results indicate that there is both a sharp edge delimiting lamin-associated regions and that sufficient information required for repositioning to the nuclear periphery resides at or inside LAD boundaries in small LASs (<2.5 kbp).

2.3.4: Cell type–specific transcription factors and proteins involved in nuclear architecture and scaffolding are involved in sequence targeting

We next aimed to determine enrichment of protein binding motifs within *Ikzf1* (A–E and G–I) LAS that would serve to identify potential protein candidates for further functional analyses. An initial search using MEME and TomTom identified YY1 (Ying-Yang 1), CTCF, and cKrox/ThPOk/Zbtb7b (as well as other BTB/POZ domain proteins)binding site motifs as among those enriched in these sequences (see Materials and methods; Figure 2.8, A and B). Not surprisingly, we also identified several binding sites for specific B cell development proteins (not depicted). Both CTCF and Zbtb7b binding site motifs were previously identified in LADs/LAS (Guelen et al., 2008; Zullo et al., 2012). Intriguingly, YY1 is known to have roles in genome regulation in many cell types, including both gene activation and repression, and has been implicated to recruit polycomb repressive complex 2 (PRC2) to chromatin to enable H3K27me3 (Satijn et al., 2001; Atchison et al., 2003; Caretti et al., 2004; Srinivasan and Atchison, 2004; Liu et al., 2007; O'Meara and Simon, 2012; Pan et al., 2013; Atchison, 2014).

We next undertook to identify all potential YY1 binding sites in LAD border regions via pair-weighted matrix motif analyses. Specifically, we analyzed the disposition of predicted YY1 and CTCF binding sites (Figure 2.8, A and B) over chromosomes 11 and 12 using MEME Suite and Genometricorr (Claeys et al., 2012; Favorov et al., 2012). Using the MEME-identified motifs, we generated maps of sites along each fragment

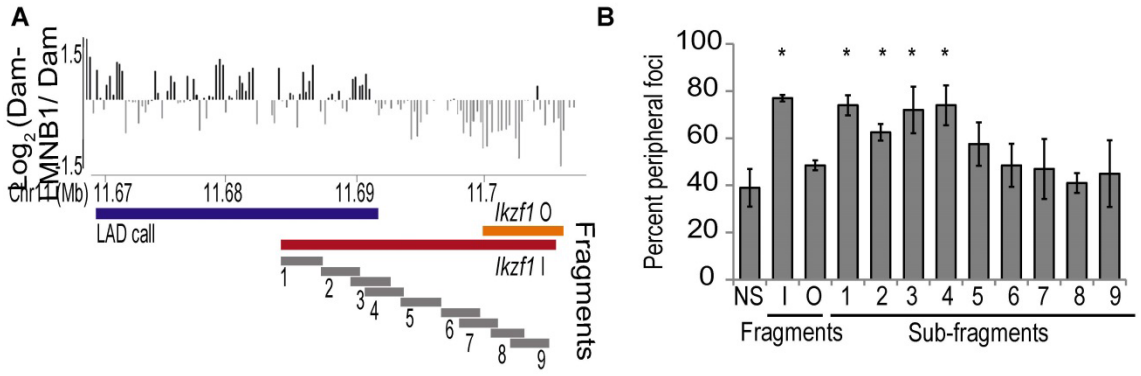


Figure 2.7: Minimal vLAD border sequences target to the nuclear periphery. (A) The lamina-targeting *Ikzf1* fragment I was further segmented (fragments 1-9) and 'switched' into TCIS clone Y. (B) Quantitation of peripheral association was determined by overlap of EGFP-LacI focus and anti-LMNB1 signal (n ≥ 50). NS is non-switched; I and O are the larger fragments (n ≥ 50). Error bars are the SD of imaging data as scored by two individuals (p≤ 0.001).

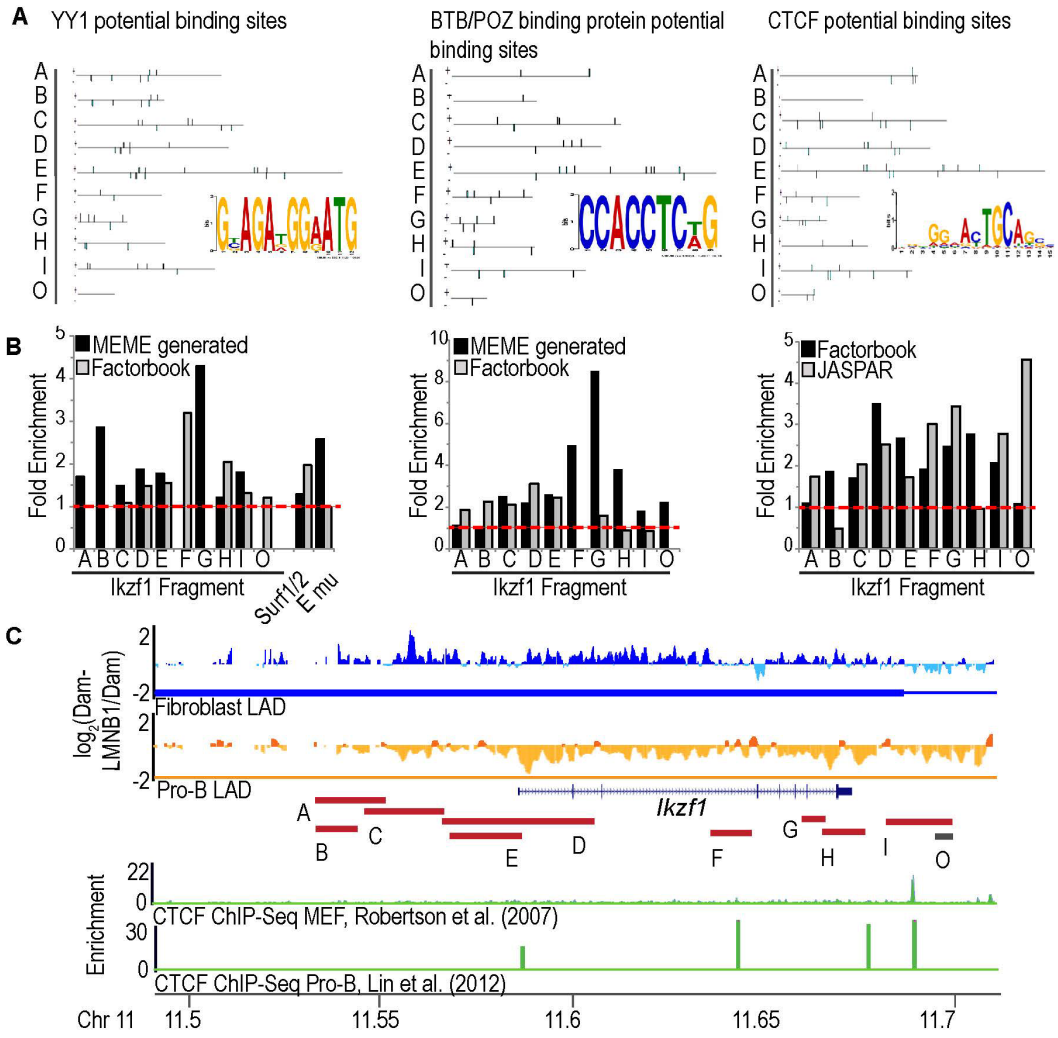


Figure 2.8: YY1, BTB/POZ type proteins and CTCF potential binding sites are enriched in LAS and at vLAD borders (A) Motif Suite analysis of LAS reveal binding motifs for YY1, BTB/POZ binding domain proteins and CTCF. The occurrence and distribution of the identified motifs (horizontal ticks) are indicated along the fragments. (B) Fold-enrichment of identified motifs in fragments (A-O, I) relative to random regions of chromosome 11 away from LAD borders from either MEME generated motifs or from the transcription factor binding motif databases Factorbook and JASPAR. For comparison, motifs on known YY1 responsive elements surrounding the Surf1/2 gene and the Emμ enhancer from the IgH locus size-matched to fragment I, are shown. (C) DamID determined traces in FB (blue) and Rag-/- proB cells (orange). FB and proB LADs are indicated by solid blue and orange bars, respectively. Ikzf1 locus (blue) is below the LADs. Peripherally targeting fragments (A-I, red) from the vLAD containing *Ikzf1* and non- targeting fragment O are in gray. CTCF ChIP-Seq data, green (show peaks at the border of the FB specific vLAD in MEFs and Rag$^{-/-}$ proB cells [Robertson, 2007 (GEO: GSE31039) and Lin, YC 2013 (GEO: GSM987805)]

using the MAST algorithm (Figure 2.8 A). In addition, because the output of MEME simply indicates a statistically significant enrichment of a given motif, we next analyzed the fold enrichment of identified motifs in our fragments relative to random regions of chromosome 11 away from LAD borders from either MEME generated motifs or from the transcription factor binding motif databases Factorbook and JASPAR (Figure 2.8 B; Sandelin et al., 2004; Wang et al., 2012; Mathelier et al., 2014). For comparison, we show enrichment of these MEME and Factorbook motifs on known YY1 responsive elements surrounding the *Surf1/2* gene (Surfeit locus protein 1 and 2) and the Emu enhancer from the *Igh* locus size matched to fragment I, for comparison (Figure 2.8 B; Cole and Gaston, 1997). Using MotifSuite, we estimate an approximately twofold enrichment of YY1 binding sites at LAD borders (±10 kb) relative to chromosomal background (unpublished data; Claeys et al., 2012). Finally, the statistical correlation package Genometricorr indicates that YY1 binding sites, either derived from our MEME analyses or from the Factorbook database of binding motifs, are more enriched relative to LAD borders compared with what would be expected by random chance ($P < 0.001$; Favorov et al., 2012).

These initial lead candidate analyses led us to next identify actual YY1 occupancy relative to LADs. Leveraging publicly available data of YY1 binding and LMNB1-DamID (e.g., LAD) maps in embryonic stem cells (ESCs) along with our data and publicly available data in proB cells, we found that high peaks of YY1 binding often correlated with LAD boundaries (Figure 2.6 A; Mendenhall et al., 2010; Peric-Hupkes et al., 2010; Vella et al., 2012). We note that in the *Ikzf1*, *Bcl11a*, and *Igh* loci, the LAD configuration in ESCs mimics those seen in FB (Figure 2.6 B, top). In addition, we note that the smaller relocating fragments (fragments 1–4), some of which, by bioinformatics analyses, were not identified as enriched in YY1 binding sites, nonetheless display YY1 occupancy in both non–B cell types, in this case ESCs, and proB cells (Figure 2.6 C). It

is thus very important to note the obvious: there are a large number of regions actually occupied by YY1 both inside and, more predominantly, outside of LAD border regions and, in the case of previously published results in ESC cells, YY1 occupancy is often associated with active promoter regions (Figure 2.6 A; Vella et al., 2012). Interestingly, although Ikzf1 is in a LAD in ESCs, it is not in proB cells, yet in both cell types, these regions have demonstrated YY1 binding (Figure 2.6 C). This likely reflects both the diversity of YY1 functions and its potential dual roles in regulating these regions. We also note, as has been previously reported, that we find CTCF binding motifs to be enriched at LAD borders (Figures 2.6 A and 2.8, A and B; Guelen et al., 2008). In all cases, the putative and determined binding sites were shown to be enriched in LAD border regions; however, it is quite evident that these regions are far from the only regions bound by these factors. Given these and previous data, we suspected that targeting to the lamina may depend upon on a combination of cell type–specific factors (such as BTB-POZ domain proteins; Figure 2.8, A and B) as well as epigenome/genome organizers, such as YY1, PRC2 components (e.g., EZH2), lamins, and/or CTCF (Figure 2.8 C). However, it was also clear from these analyses that simply trying to determine bioinformatically (motif or occupancy analyses) the roles of these proteins in LAD establishment would be problematic.

To more rigorously determine the role of the identified candidate proteins in LAD establishment and/or maintenance, we used a targeted shRNA-mediated knockdown strategy (Figure 2.9). Specifically, a test fragment from the *Ikzf1* vLAD border (LAS I) and a control fragment from the *Igh* vLAD (LAS N) were introduced into TCIS insertion sites in clone 12 and Y, and these same cells were then subjected to shRNA-mediated knockdown of either proteins previously identified to be important for de novo lamina association (Zbtb7b, HDAC3, and Lap2β) as well potential novel players based on the

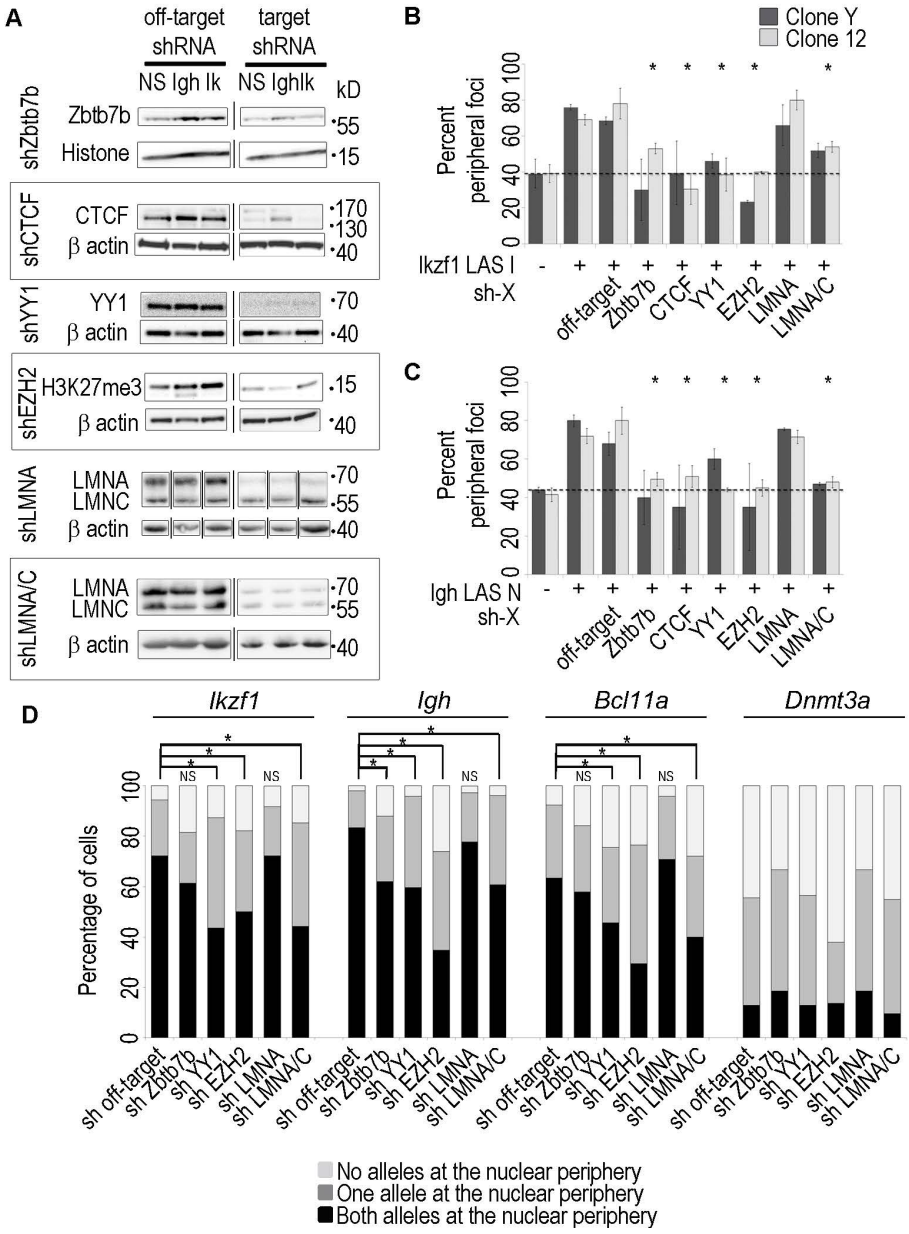


Figure 2.9: Knockdown of Zbtb7b, CTCF, YY1, EZH2 or LMNA/C leads to a loss of peripheral association of LAS. (A) Immunoblot analysis of protein lysates from TCIS lines Y and 12, harboring LAS *Ikzf1* (I) or *Igh* (N) and control 'non-switched' (NS) with either off-target or target shRNA as indicated to the left of each blot. (B, C) Quantitation of IF testing the disposition of *lacO* and LMNB1 in TCIS clones harboring the (B) *Ikzf1*(I) or (C) *Igh* (N) LAS, after 4d treatment with indicated shRNA (n≥50, P≤ 0.001). (D) Quantitation of 3D DNA ImmunoFISH for BAC probes to the indicated endogenous LAD regions in MEFs treated with indicated shRNA constructs (n ≥ 50, p≤ 0.05).

literature (LMNA/C) or in this study (CTCF, YY1, and the PRC2 component EZH2). We determined the disposition of the LAS before shRNA-mediated knockdown with either an off-target shRNA control or with the indicated protein target (Figure 2.9). Lap2-β and HDAC3 shRNA mediated knockdowns led to substantial cellular death and were, therefore, not able to be analyzed with confidence; however, the remainder of the constructs appeared to have little effect on cell viability within the time frame used by our assays (Figure 2.10). Interestingly, all of these knockdowns, with the prominent exception of LMNA, led to the loss of peripheral association of targeting fragments (Figure 2.9, B and C, $P < 0.05$). It is important to note that the LMNA knockdown construct specifically knocks down LMNA, whereas the LMNA/C construct knocks down both LMNA and LMNC (Figure 2.9, B and C). Given these results, it is tempting to speculate that LMNC, but not LMNA, is important for maintaining lamina-proximal positioning. We next asked whether the proteins demonstrated to impact LAS-directed targeting to the nuclear lamina also affected endogenous LAD organization. Both LMNA/C and YY1 perturbation reduced the association of endogenous LADs containing *Ikzf1, Igh,* and *Bcl11a* with the nuclear lamina as determined by 3D immuno-FISH (Figure 2.9 D, $P < 0.005$). In contrast, disruption of Zbtb7b only affected the association of the *Igh* locus with the lamina with any degree of statistical significance (Figure 2.9 D). Collectively, these data suggest that peripheral targeting and maintenance of lamina association of these regions is dependent on both LMNA/C and YY1. Again, ablation of LMNA alone had no effect on the disposition of endogenous loci. Because YY1 shRNA-mediated knockdown affected the nuclear disposition of both ectopically recruited TCIS sites and endogenous loci (Figure 2.9), we specifically tested whether YY1 is sufficient to target an ectopic site to the nuclear periphery. To enable such an experimental test, we expressed a YY1-EGFP-LacI fusion protein in our clones Y and 12 (harboring the original non-relocating vector sequence) in the presence or absence of IPTG, thus

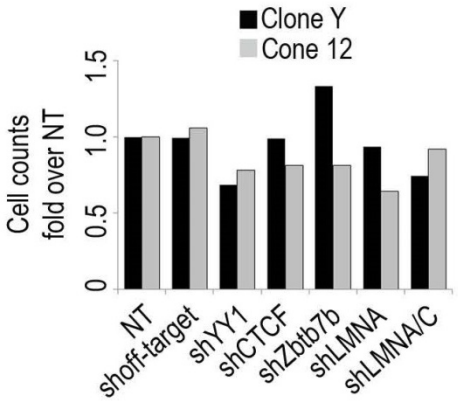


Figure 2.10: Cell survival after 4 days of shRNA treatment (A) Cell survival, as expressed by cell counts of treated cells over non-transduced (NT) after 4 day treatment of shRNA constructs. Data shown are from a single experiment

allowing for regulated accumulation of YY1 at the integration site (Figure 2.11, C and D). The YY1-EGFP-LacI fusion protein displayed the same punctate distribution in the nucleus as endogenous YY1, making identification of the bound *lacO* array difficult in most cells (Figure 2.12 A). We note, however, that some cells express the fusion at lower levels and were thus able to be assayed by IF; in these cells, the YY1-EGFP-LacI loci collocate with YY1-enriched foci (Figure 2.12 B). To enable clear identification of the disposition of these YY1-bound TCIS insertions, we performed 3D immuno-FISH and measured their association with thee nuclear periphery using LMNB1 to demarcate the nuclear periphery (Figure 2.11, C and D). Although these integrations are centrally disposed when bound by EGFP-LacI, nearly 80% of the YY1-EGFP-LacI foci are at the nuclear periphery after 24 h. This finding is also borne out by DamID analyses of YY1-bound TCIS loci (Figure 2.11 E, *Ikzf1* (I) or -IPTG, black bars) compared with controls (Figure 2.11 E, nonswitched and +IPTG, black bars). We note that LAS I switched into TCIS also displays lamina association by DamID (Figure 2.11 E). In these experiments, relative lamina association and efficacy of the DamID protocol was monitored in the same experiment by PCR using *Igh* primers internal to the Igh vLAD. Specificity of the protocol was measured by using primers to detect a region in a small “dip” between and proximal to LAD regions (Figure 2.11 E; Wen et al., 2012). Importantly, simply overexpressing YY1 does not lead to repositioning of ectopic TCIS integrations (without recruitment to the *lacO* array, ±IPTG) or the control (dip) region to the lamina, indicating that simply overexpressing YY1 does not cause global reorganization to the nuclear lamina or even spreading into LAD proximal regions (dip; Figure 2.11). An accumulation of the YY1 protein, at a discrete site, is therefore sufficient to target to the nuclear periphery. In contrast, tethering of Zbtb7b is not sufficient to drive these regions to the nuclear periphery, even though the fusion protein is expressed, recruited to TCIS, and

Figure 2.11: YY1 targets chromatin to the nuclear periphery. (A) Expression of YY1 and Zbtb7b fusions to EGFP-LacI were verified by immunoblot by detecting with α-GFP (B) qRT-PCR of Col1A1 and fibronectin in FB cells overexpressing Zbtb7b-EGFP-LacI. (C) Representative images of 3D DNA-ImmunoFISH. EGFP-LacI, YY1-EGFP-LacI or Zbtb7b-EGFP-LacI fusion proteins detected by α-LacI (magenta, arrowhead) co-localize with the TCIS insert site (*lacO* probe, green, arrowhead) and LMNB1 (red). (D) Quantitation of 3D DNA ImmunoFISH for *lacO*/LacI position in two TCIS clones [$n \geq 50$, ($p \leq 0.001$)]. (E) qPCR analysis of Dam-LMNB1/Dam in clone Y and 12 under the indicated conditions. A value >1 for [Dam-LMNB1/ Dam only] indicates enrichment. Primers to the TCIS locus, the *Igh* locus (+ control) and a non-lamina associated region, dip (- control), were used for amplification. The data shown are from a single representative experiment. (F) Representative images of 3D DNA-ImmunoFISH in clone Y containing either *Ikzf1 (I)* LAS or YY1-EGFP-LacI (+/- IPTG). LMNB1 (red), γ-satellite (cyan), *lacO* probe (green) and Hoechst (blue). (G) Quantitation of 3D DNA ImmunoFISH for *lacO* position in relationship to both the nuclear lamina and γ-satellite DNA with either *Ikzf1(I)* LAS integrated or YY1 bound ($n \geq 50$, $p \leq 0.001$). (H) Quantitation of the disposition of *lacO* sites by 3D ImmunoFISH analysis in clone 12 and Y overexpressing YY1-EGFP-LacI(-IPTG) before and after shRNA mediated knockdown (as indicated, $n \geq 50$, $p \leq 0.001$).

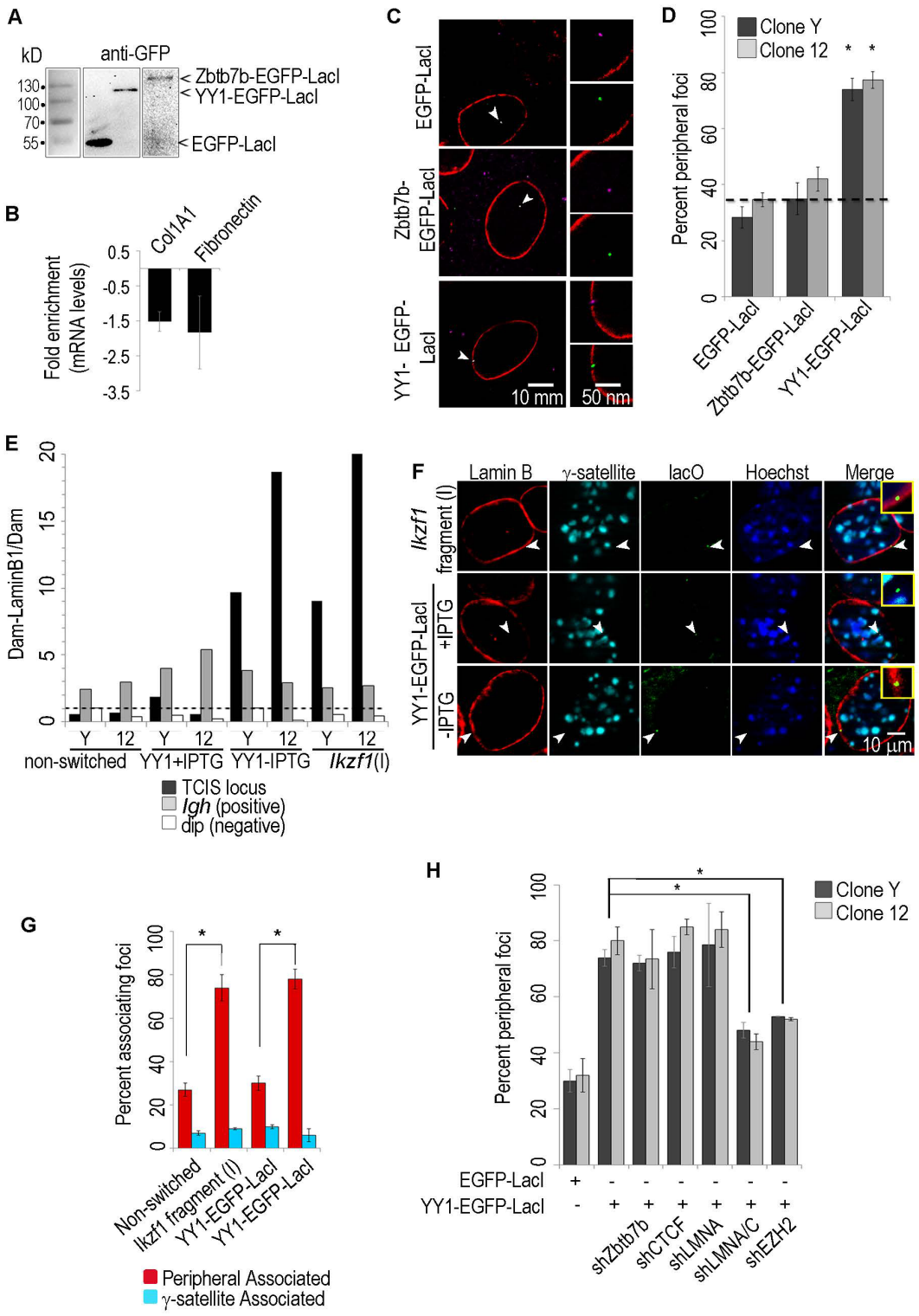


Figure 2.11: YY1 targets chromatin to the nuclear periphery.

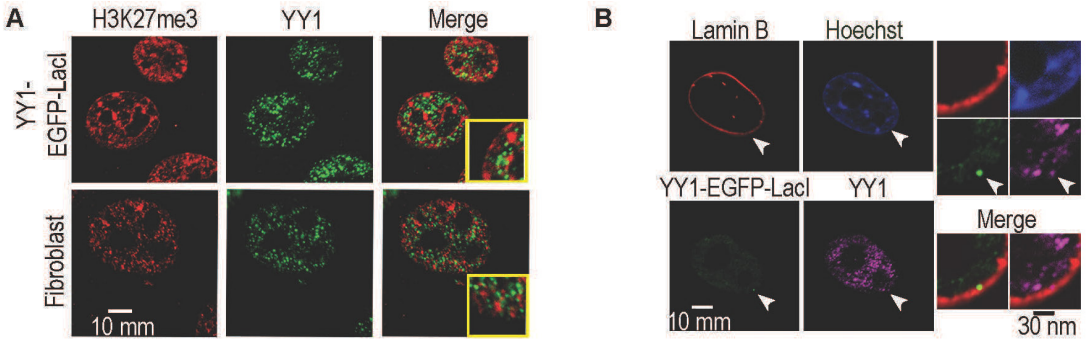


Figure 2.12: Endogenous and overexpressed YY1 in fibroblasts (A) IF using α-YY1 in either wild-type or YY1-EGFP-LacI expressing FB (green). YY1 is punctate in cells and does not co-localize to H3K27me3 signal (red). Inset is 300X magnification. (B) YY1-EGFP-LacI, when expressed in a FB TCIS clone, leads to *de novo* LAD formation. LMNB1 (red), YY1-EGFP-LacI (green), Hoechst (blue) and YY1 (magenta). Arrowhead indicates the clearly identifiable YY1-EGFP-LacI foci at the *lacO* array of the TCIS.

appears to be functional, as assayed by reduction in col1a1 (collagen, type I, α 1) and fibronectin gene expression (Figure 2.11, A–D; Widom et al., 1997; Renard et al., 2008). Interestingly, YY1 is not normally enriched at the nuclear lamina, although we do detect faint rim staining upon longer exposure when detecting endogenous YY1 by IF (Figure 2.13). However, we note that although YY1 is not obviously enriched at the nuclear lamina in most cells, H3K27me3 and Hoechst-stained heterochromatin is indeed enriched in the region underlying the lamina, enhanced in those cells overexpressing YY1 (Figure 2.12 A, B).

Interestingly, there are previous studies indicating that recruitment of YY1 leads to association with pericentromeric heterochromatin (Shestakova et al., 2004; Josse et al., 2012). We therefore asked whether directed recruitment of YY1 led to the assays to determine the amount of colocalization of the YY1- bound TCIS locus with association of the TCIS site with these domains by using either 3D immuno-FISH or IF either pericentromeric γ -satellite DNA or with the centromeric protein CENP-A (Figure 2.11, F and G; and Figure 2.14, A and B). We observe no increase in association with either of these subnuclear regions. Collectively, these data indicate that YY1 is able to direct recruitment of an ectopic site to the nuclear lamina and that this recruitment is independent of association with pericentromeric or centromeric heterochromatin domains. Given that YY1 recruitment to TCIS is sufficient to target that region of the genome to the nuclear lamina, we next asked whether such repositioning was dependent on putative YY1 interactors and/ or lamina proteins. Both CTCF and BTB-POZ proteins have been implicated to interact with YY1 (Donohoe et al., 2007; Zlatanova and Caiafa, 2009; Boulay et al., 2012). shRNA-mediated knockdown of LMNA/C was able to prevent or disrupt YY1-mediated tethering to the nuclear lamina, although LMNA knockdown alone was not sufficient to cause disruption of this interaction (Figure 2.11 H). In contrast, knockdown of either Zbtb7b or CTCF did not

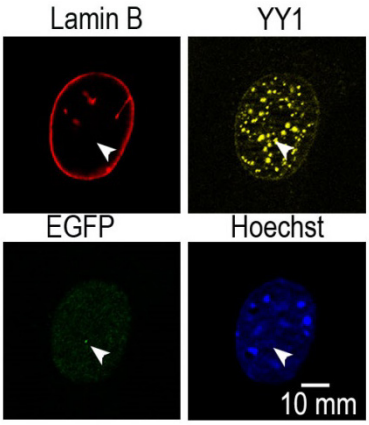


Figure 2.13: Endogenous YY1 shows a faint rim stain at the nuclear periphery. Endogenous expression of YY1 (yellow) in TCIS clone Y expressing EGFP-LacI (green), LMNB1 (red), and Hoechst (blue). YY1 is over-exposed to show nuclear rim staining.

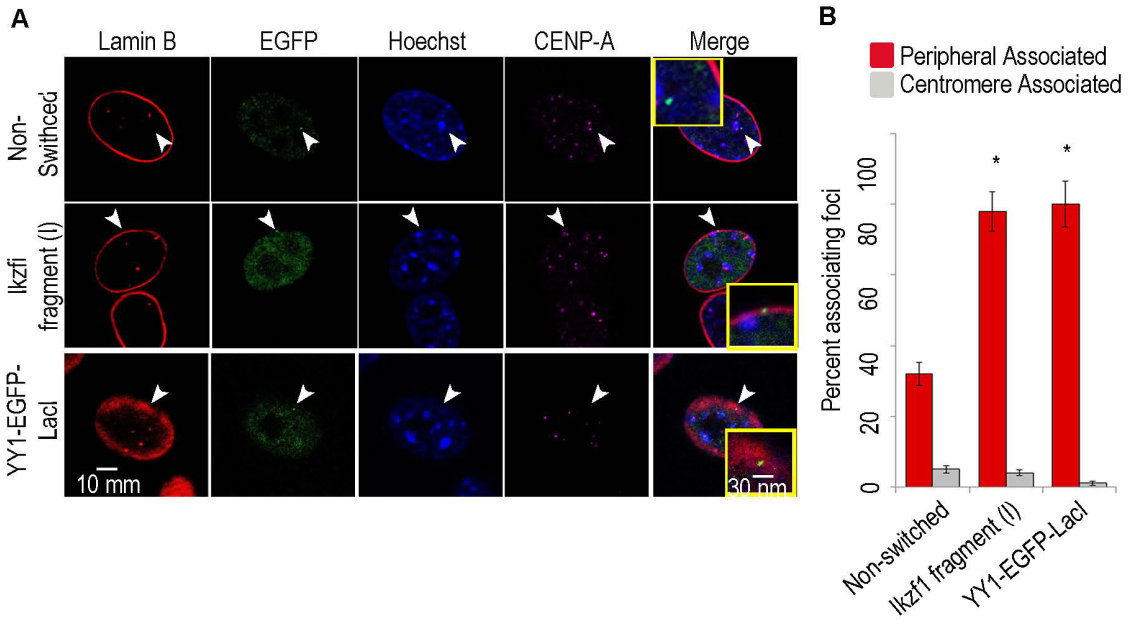


Figure 2.14: Fragment or YY1 driven *de novo* LADs do not associate with centromeric heterochromatin. (A) Representative images of 3D DNA-ImmunoFISH in TCIS clones containing either a switched fragment I, or harboring YY1-EGFP-LacI in the presence and absence of IPTG. Lamin B (red), EGFP-LacI (green), CENP-A (magenta) and Hoechst (blue). (B) Quantitation of Immunofluoresnce for EGFP-LacI association with either LaminB1 or CENP-A in TCIS clone Y ($n \geq 50$, $p \leq 0.001$).

disrupt peripheral association, indicating either that these proteins are not involved in this process or that YY1 recruitment is able to bypass their functional requirements (Figure 2.11 H). Nonetheless, these data show that YY1-mediated recruitment to the nuclear lamina depends on LMNC and, possibly, LMNA/C levels.

Intriguingly, H3K27me3 has been reported to be enriched at LAD borders but not LAD interiors (Guelen et al., 2008). Using publicly available data identifying H3K27me3 in MEFs (GEO accession no. GSE48649), we also note that there is a striking enrichment of H3K27me3 at the border of the LADs containing the *Ikzf1*, *Igh*, and *Bcl11* a loci and more generally at LAD borders (Figure 2.15, A and B; and Figure 2.6 A; Simon et al., 2013). These H3K27me3 modifications overlap with our relocating fragments (Figure 2.15 C). We next asked whether YY1 recruitment to the TCIS locus mirrored what is observed at endogenous LAD borders, namely the accumulation of H3K27me3-modified chromatin at the insert site (Figure 2.15, B and C). By chromatin immunoprecipitation (ChIP), we detected strong enrichment of H3K27me3 upon recruitment of YY1 and found, additionally, that our inserts harbor H3K9me2/3 modifications independent of YY1 recruitment (Figure 2.15 D). We note that the antibodies used for detecting the H3K9 methyl modifications have significant cross-reactivity between dimethyl and trimethyl variants, and so, we describe our data in terms of H3K9me2/3 (Peach et al., 2012). Intriguingly, a recent study indicated that H3K9 methyl status may determine chromosomal positioning at the lamina of ectopic insertion sites (Bian et al., 2013). In addition, genome-wide analyses in *C. elegans* have shown a correlation between H3K9me1/2/3 enrichment and LAD regions (Towbin et al., 2012). Moreover, H3K9me2 has been previously reported to play a role in LAD organization and in regulation of the *Igh* locus, and we also detected enrichment of this modification

Figure 2.15: H3K27me3 enriched at endogenous LAD borders and YY1-bound TCIS. (A, B) H3K27me3 enrichment at LAD borders (Simon, 2013, GSE48649). The entire FB LAD is shown for *Ikzf1* and *Igh* to enable visualization of both borders and blue bar underscores LAD region determined by LAD histograms [log_2(Dam-LMNB1/Dam)]. The genes are shown (blue) and the *Ikzf1* vLAD border region tested in this study is highlighted in yellow. Primers used for ChIP to the *Igh* locus are indicated (orange). (C) Magnification of the 20kb edge of the FB specific vLAD containing *Ikzf1*. LAS (magenta) and non-targeting fragments (gray) are shown above H3K27me3 histograms. (D) ChIP analysis of YY1-recrutied to the TCIS locus (+/- IPTG). Enrichment of H3K9me2, H3K9me3 and H3K27me3 at the indicated locus is shown as a percent of input.

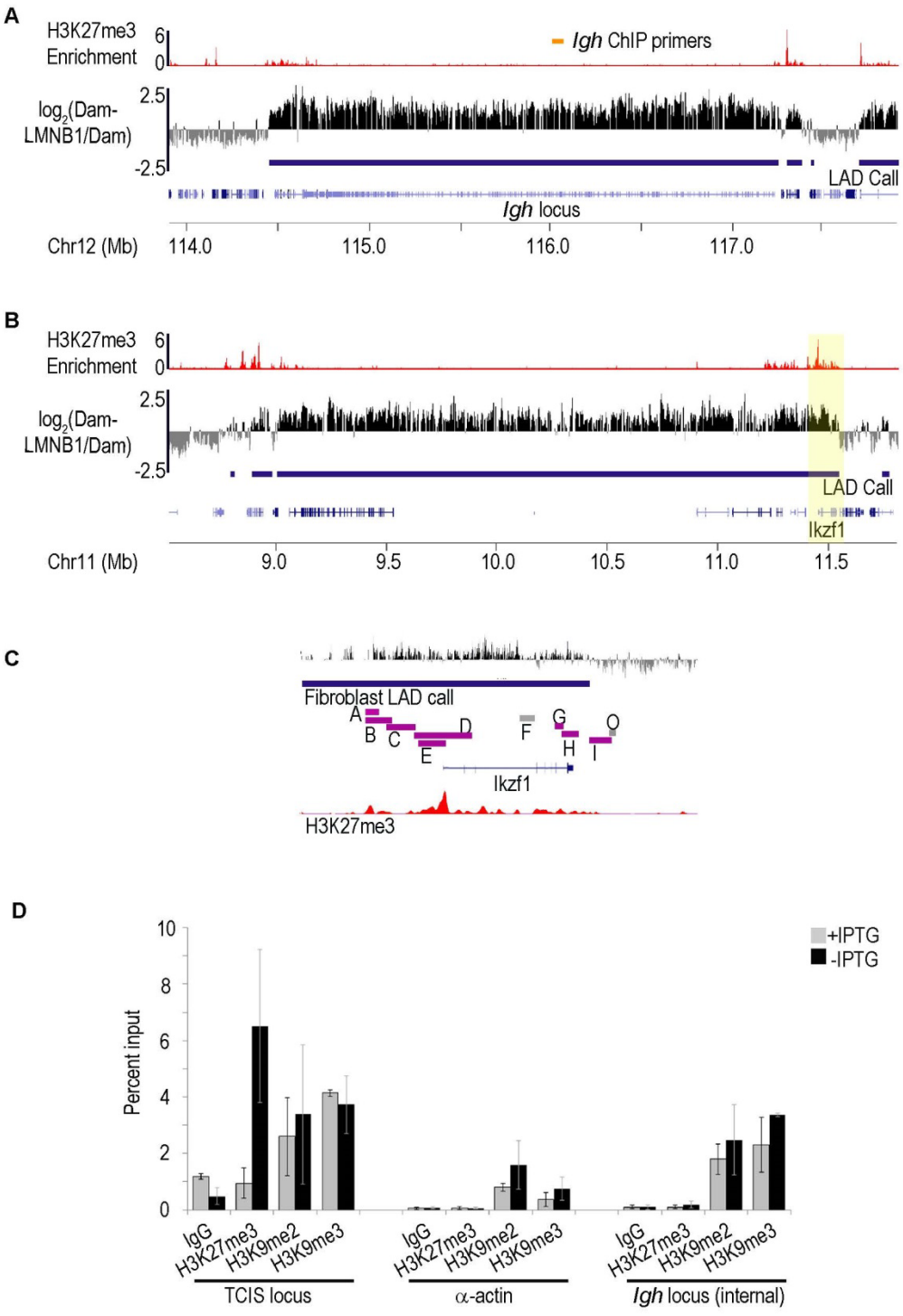


Figure 2.15: H3K27me3 enriched at endogenous LAD borders and YY1-bound TCIS.

in the interior of the LAD comprising the *Igh* locus (Figure 2.15 D; Johnson et al., 2004). As expected, these more interior primers of the *Igh* LAD did not detect an enrichment of H3K27me3, indicating that this modification is present at border regions of LADs, but not the interior, in agreement with our and others' analyses of H3K27me3 ChIP-seq signatures relative to LAD borders (Figures 2.6 A and 2.15 D; Guelen et al., 2008). We therefore conclude that our YY1-bound TCIS system likely recapitulates the endogenous chromatin state of LAD borders in that they have both H3K9me2/3 and H3K27me3 modifications.

YY1 has been shown to act as both a negative and positive transcriptional regulator (Thomas and Seto, 1999); however, one reported role is in recruiting PRC2 to mediate transcriptional repression (Basu and Atchison, 2010; O'Meara and Simon, 2012; Basu et al., 2014). Previously, we showed that reduction in the PRC2 histone methyltransferase component EZH2, which trimethylates histone H3 on lysine 27, led to the loss of peripheral association of a LAS fragment (Figure 2.9). In addition, disruption of EZH2 prevented YY1-mediated recruitment to the nuclear lamina, indicating that H3K27me3 is necessary for this repositioning as well (Figure 2.11 H). We note, however, that overexpression of YY1 concomitant with disruption of EZH2 in the same cells led to a substantial decrease in cell viability (unpublished data). As a separate test to determine whether H3K27me3 was required for the YY1-mediated repositioning, we treated TCIS clones with either relocating LAS fragments recombined into the locus (Figure 2.9) or bound by YY1 (Figure 2.11) with 3-deazaneplanocin (DZNep). DZNep is an indirect inhibitor of S-adenosyl-methionine–dependent methylation that disrupts EZH2 function, thus blocking H3K27me3 deposition. In addition, there are reports that, depending on the study, implicate DZNep in the reduction of H3K4me3, H4K20me3, and at higher concentrations, H3K9me3 (Figure 2.16 A; Fiskus et al., 2009; Miranda et al., 2009; Lee and Kim, 2013). We used DZNep in addition to shRNA-mediated knockdown

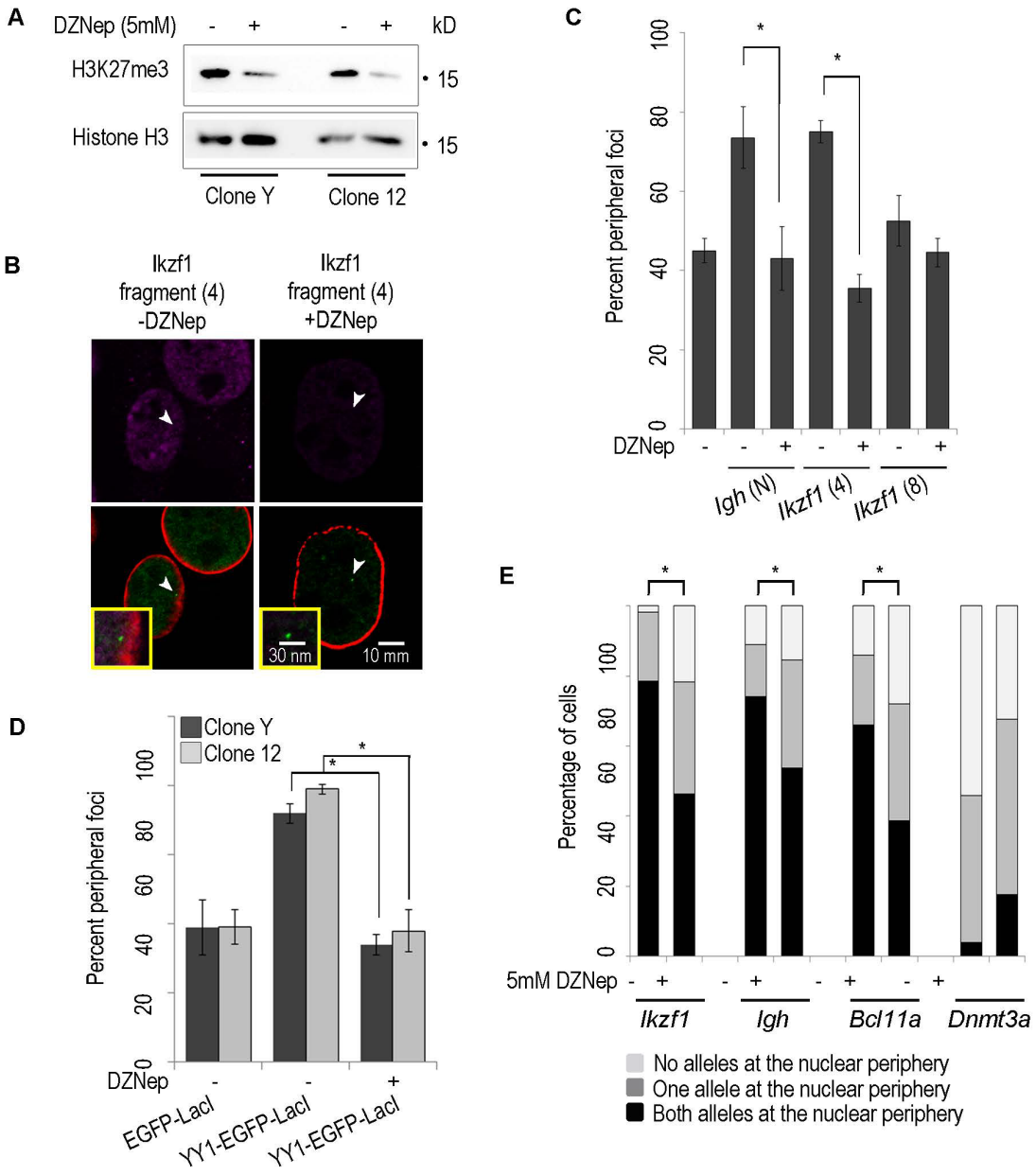


Figure 2.16: DZNep treatment leads to loss of peripheral targeting. (A) Immunoblot analysis of H3K27me3 in clones 12 and Y +/- 5μM DZNep treatment for 24h. (B) Representative images of clone Y harboring the Ikzf1 (4) LAS, +/- DZNep treatment. EGFP-LacI foci (green, arrowhead) are shown relative to LMNB1 (red) and H3K27me3 (magenta). (C) Quantitation of IF for DZNep treatment of clone Y harboring Igh (N) and Ikzf1 (4) LAS and a non-targeting fragment Ikzf1 (8), n ≥ 50, p≤0.05. (D) Quantitation of IF of 24h, 5μM DZNep treated clones Y and 12 expressing EGFP-LacI or YY1-EGFP-LacI, n ≥ 50, P≤0.001. Error bars are SD. (E) Quantitation of 3D DNA ImmunoFISH for the indicated endogenous loci in MEFs treated with 5μM DZNep (24h) MEFs, n ≥ 50, p≤0.05.

of the EZH2 methyltransferase because loss of EZH2 might lead to destabilization of some of the YY1 containing complexes, thus making results from such an experiment difficult to interpret. We note that treatment with 5 μM DZNep resulted in modest effects on cell proliferation at 24 h of treatment (the time point used for our assays), but the cells appeared to be otherwise healthy (Figure 2.17). Strikingly, lamina association driven by LASs are disrupted when treated with DZNep (Figure 2.16, B and C) as they are with EZH2 knockdown (Figure 2.9, B and C), indicating that their repositioning is dependent on H3K27me3. Intriguingly, DZNep treatment affected all LAS fragments similarly, including the *Igh*(N) LAS, indicating that either H3K27me3 is involved in initiating lamina association regardless of LAS origin or that our treatments also affected other histone modifications necessary for repositioning, especially H3K9me2/3 (see following paragraph; Figure 2.16 C; Towbin et al., 2012; Bian et al., 2013). However, in support of a strong role for H3K27me3 in LAS-directed repositioning, shRNA-mediated knockdown gave similar results for these same LAS fragments (Figure 2.9 C). Similarly, DZNep treatment of either YY1-bound loci or endogenous LADs resulted in their movement away from the nuclear lamina, again similar to our findings with shRNA-mediated knockdown of EZH2 (Figure 2.16, D and E). Collectively, these results suggest that H3K27me3 is necessary to target to and maintain proximity to the nuclear lamina. As previously mentioned, there are studies in the literature implicating histone H3K9me2/3 modifications in recruitment of domains to the nuclear lamina. We also note that our TCIS system harbors H3K9me2/3-modified chromatin, as has been previously reported for these types of arrays (Janicki et al., 2004; Towbin et al., 2012; Bian et al., 2013).

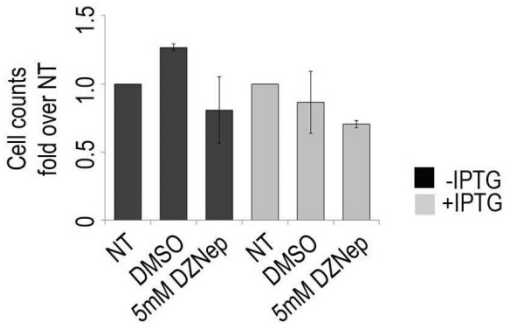


Figure 2.17: Cell survival with Bix-01294 treatment. Cell survival, as expressed by cell counts of treated cells over non-transduced (NT) after 24 hour treatment of DMSO (vehicle) and 5μM Bix-01294.

Taking these findings into consideration, we next asked whether the association with the nuclear lamina might be dependent on both types of modifications—a potential bivalent heterochromatin signal. Intriguingly, H3K9me2/3 modifications appear to be broadly distributed across LAD domains (unpublished data; Towbin et al., 2012), although H3K27me3 modifications appear to be restricted (relative to LADs) at the border regions (Figure 2.15; Guelen et al., 2008). These border regions may represent regions where the genome is constantly reestablishing LAD boundaries as indicated by the preponderance of active promoters immediately flanking LADs (Guelen et al., 2008). To test whether repositioning of ectopic regions to the nuclear lamina requires H3K9me2/3 modifications, we used the inhibitor BIX-01924 to block histone dimethylation at lysine 9 of H3 (Kubicek et al., 2007; Bian et al., 2013). We chose to use this inhibitor because, despite our best efforts, we were unable to find an shRNA construct for G9a (the methyltransferase responsible for H3K9me2; Lachner and Jenuwein, 2002) that worked without significant cell death (unpublished data). Western blots show that treatment with 5 μM BIX-01924 leads specifically to inhibition of H3K9me2 but not H3K9me3 (Figure 2.18, A and B). Initially, we subjected cells harboring our TCIS system with the *Ikzf1* (I) LAS or with YY1 bound to treatment with the inhibitor for 24 h, and, although we saw significant down-regulation of H3K9me2, we were unable to reliably detect loss of lamina association (Figure 2.18, C and D). Interestingly, another study also tried using BIX-01924 to disrupt lamina associations through down-regulation of H3K9me2 but also were unable to detect loss of association; however, the authors noted that their assays to detect lamina association were designed to detect within 0.5 μm of the nuclear lamina, and therefore, they may have missed a more subtle loss of association (Bian et al., 2013). Another study clearly implicated this

Figure 2.18: H3K9me2/3 is necessary for *de novo* LAD formation. (A, B) Immunoblot analysis of protein lysates from TCIS lines either harboring *Ikzf1 (I)* LAS or bound by YY1-EGFP-LacI (-IPTG) and control, 'non-switched'. Cells were treated with either vehicle (DMSO) or 5μM Bix-01294 for 24 or 60h. Proteins were detected by the antibodies indicated; loading control is total histone H3. (C) Representative images of clone Y harboring the *Ikzf1 (I)* LAS +/-5μM Bix-01294 after 24 or 60h treatment. EGFP-LacI foci (arrowhead), LMNB1 (red) and Hoechst (blue). Insets, 300X. (D) Quantitation of the disposition of the TCIS insert for vehicle and 5μM Bix-01294 treatment at 24 and 60h treatment of clone Y harboring *Ikzf1(I)* LAS and YY1-EGFP-LacI (-IPTG, $n \geq 50$). (E) qPCR analysis of DamID in clone Y harboring *Ikzf1*(I) LAS, +/- 5μM Bix-01294 (60h). A value >1 of, Dam-LMNB1 over Dam only, indicates enrichment of the tested locus. Positive and negative controls are primers to an internal region of the *Igh* locus and a non-lamina associated region, dip, as previously. The data shown are from a single representative experiment. (F) Quantitation of 3D DNA ImmunoFISH for the indicated in 48h, 5μM Bix-01294 treated MEFs ($n \geq 50$, $p \leq 0.01$). (G) Immunoblot analysis of protein lysates from TCIS clone Y harboring *Ikzf1 (I)* LAS or YY1-EGFP-LacI (-IPTG) treated with indicated shRNAs. Proteins were detected by the antibodies indicated; loading control is total histone H3. (H) Quantitation of the disposition of TCIS sites of clone Y harboring *Ikzf1 (I)* LAS or bound by YY1-EGFP-LacI treated shRNAs as indicated (48h, $n \geq 50$).

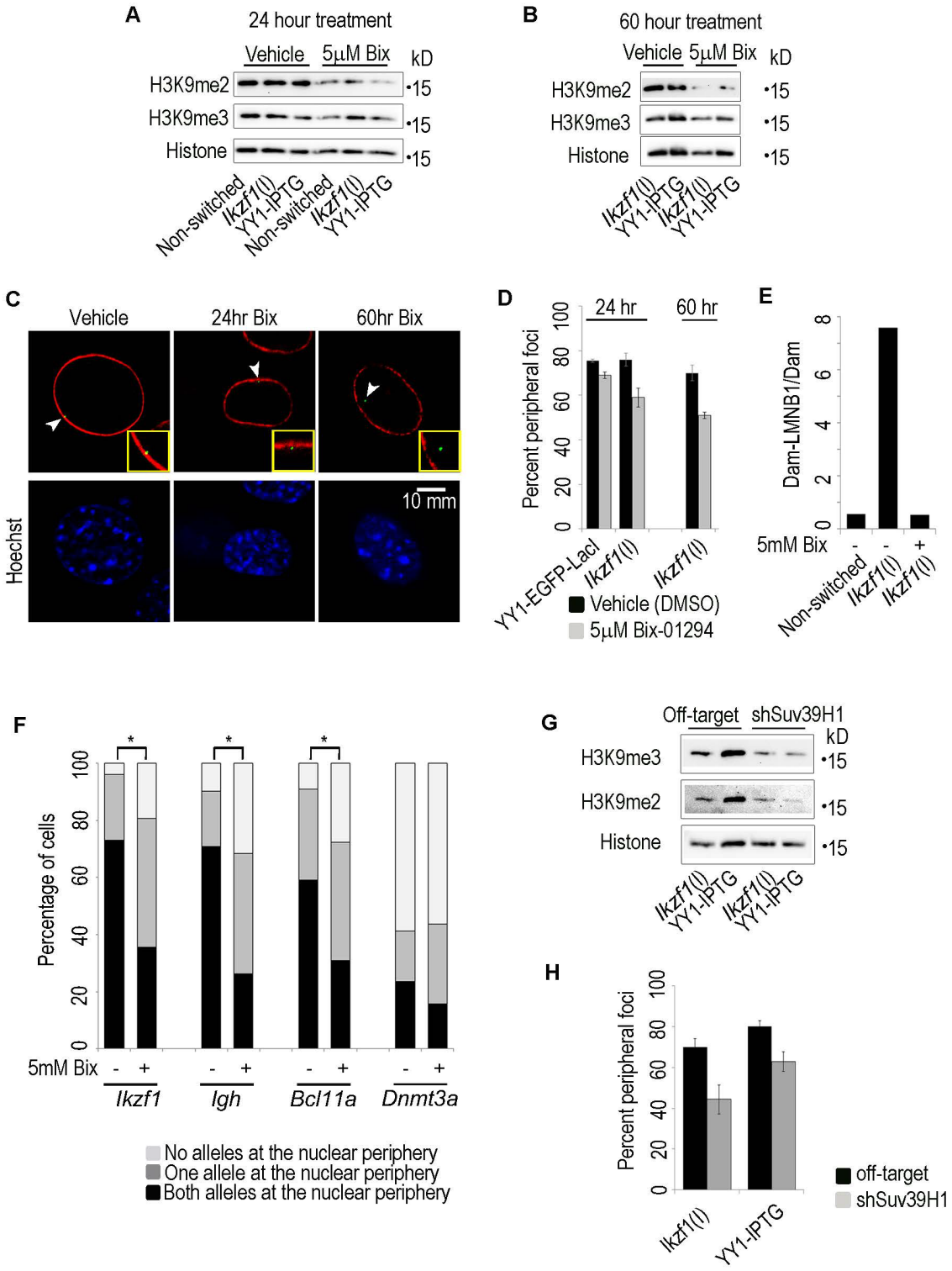


Figure 2.18: H3K9me2/3 is necessary for *de novo* LAD formation.

modification with recruitment to the lamina (Towbin et al., 2012). We noted a slight, but not statistically significant, loss of association of our targeting fragments but were struck by a qualitative difference in the lamina association.

Because of these conflicting studies in the literature and our somewhat ambiguous cytological data, we decided to repeat this experiment to enable both cytological and DamID analyses. Specifically, we treated cells harboring relocated TCIS sites with BIX-01294 for 12 h and then transduced these cells with Dam or Dam-LMNB1 and harvested after 48 h (total of 60 h). We noted some slowdown of cell division at this time point, but the cells were healthy and still undergoing division (unpublished data; Kubicek et al., 2007). At this time point, there is statistically significant repositioning away from the nuclear lamina, but at a relatively low frequency (Figure 2.18 D). However, by using DamID to measure lamina association, there was, quit strikingly, a complete loss of association with the nuclear lamina (Figure 2.18 E). We tested whether BIX-01294 treatment affected endogenous loci as well and chose to use an intermediate time point (48 h). The endogenous loci, in contrast to our TCIS relocated fragments, show appreciable loss of association (Figure 3.18 F). We next queried whether H3K9me3 affected our loci in the same way by using an shRNA-mediated knockdown of the SUV39H1 (Suppressor of Variegation 3–9 homologue 1), a methyltransferase that mediates H3K9me3 (Figure 2.18, G and H; Lachner and Jenuwein, 2002). Although SUV39H1 knockdown did disrupt localization of both the LAS- and YY1-directed lamina association, we were unable to attribute this directly to H3K9me3 because removal of SUV39H1 also perturbed H3K9me2 levels (Figure 2.18 G). Although we found this result somewhat surprising, we note that loss of SUV39H1 has previously been shown to disrupt H3K9me2 (Murayama et al., 2008). Nonetheless, these data provide compelling evidence that both H3K27me3 and H3K9me2/3 are required for targeting an ectopic site to the nuclear lamina.

2.4: Discussion

Our results implicate specific DNA sequences within LADs and chromatin state, specifically H3K27me3 and H3K9me2/3, in establishing de novo lamina associations. In addition, we suggest a role for levels of A-type lamins and, specifically, LMNC in regulating this repositioning (Figure 2.19). Our work focused on LAD border regions. We show that some LASs are sufficient for directing an ectopic site to the nuclear lamina, and this repositioning is dependent on YY1 (Figure 2.9) and H3K27me3 (Figures 2.9 and 2.16), a histone modification demonstrated to be enriched in these regions at endogenous LADs (Figures 2.16 and 2.6 A; Shah et al., 2013). We demonstrate, in agreement with two previous studies, that directed association with the nuclear lamina is also dependent on H3K9me2/3 (Figures 2.18; Towbin et al., 2012; Bian et al., 2013). In addition, a recent study, using a human fibrosarcoma cancer cell line (HTC75), demonstrated that using either BIX-01294 or G9a shRNA-mediated knockdown to perturb H3K9me2 resulted in loss of LAD association with the nuclear periphery (Kind et al., 2013). Recent work in our laboratory also shows that these modifications are necessary for functional organization of a chromosomal territory (unpublished data). Thus, we speculate that these LAD border regions, which are enriched in both H3K27me3 and H3K9me2/3, constitute a targeting signal for LAD formation (Figure 2.19). Intriguingly, recent studies have also identified specific nuclear envelope transmembrane proteins as involved in organizing and regulating chromatin at the nuclear periphery (Reddy et al., 2008; Zuleger et al., 2013; Wong et al., 2014). We

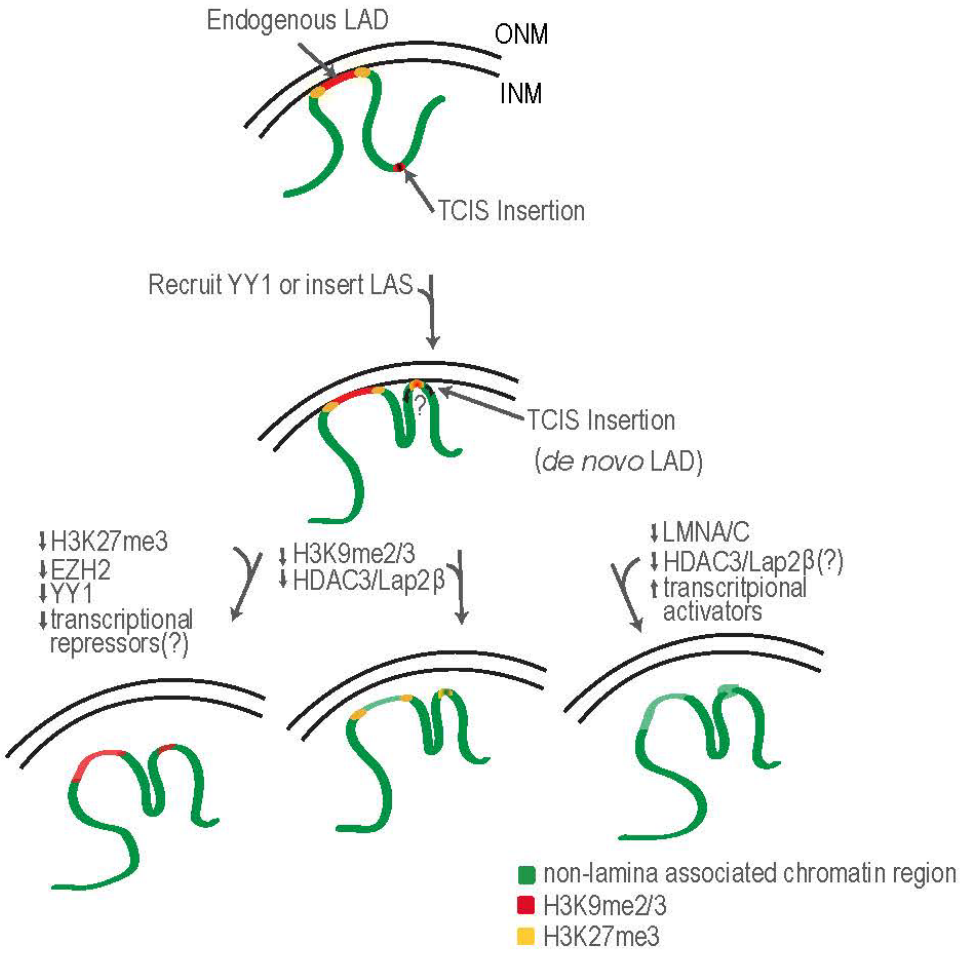


Figure 2.19: A model for directed reorganization of chromatin. A cross-section of a portion of the interior of a FB nucleus shows chromatin as a lamina associated domain (LAD, red with yellow borders) or a non-lamina associated region (green). An ectopic insertion of a LAS into the TCIS insert site is poised for relocation to the lamina due to H3K9me2/3 modifications present on the insert (red). Acquisition of H3K27me3 (yellow) leads to lamina association. Abrogation of H3K27me3 by knock-down of EZH2 or specific transcriptional repressors or by drug inhibition, leads to loss of peripheral targeting at both endogenous and ectopic loci. Our data suggests that K27me3 precedes movement to the lamina. Abrogation of H3K9me2/3 leads to a loss of association of endogenous and ectopic loci, as has also been shown by previous studies (Bian, 2013, Towbin, 2013, Kind 2013). We propose that these two facultative heterochromatin marks, H3k27me3 and H3k9me2/3, cooperate (bi-valency) to drive LAD formation and/or maintenance. Intriguingly, we also uncovered a potential role for LMNC (or LMNA/C levels) in this process and disruptions in LMNA/C have been linked to global loss of H3K27me3 as well as H3K9me3 (Luperchio, 2013; Wong, 2014).

therefore propose a model for regulation of vLADs that incorporates cell type–specific transcription factors, chromatin modifiers and organizers, and nuclear lamina proteins to facilitate cell type–specific genome organization (Figure 2.19). The work described herein focused on functional tests of LAD organization at LAD borders. In agreement with a previous study, we find that GA-rich sequence motifs, potentially bound by BTB-POZ domain proteins, are overrepresented in lamina relocating fragments but also find an enrichment of YY1 and CTCF binding sites (Figures 2.6 B, 2.7 and 2.8). The presence of CTCF binding sites was not unexpected as a result of its known enrichment at the boundaries of LADs (Vogel et al., 2007; Guelen et al., 2008; Handoko et al., 2011). As a previous study has indicated that robust YY1 binding often requires a cofactor (Golebiowski et al., 2012), we speculate that Zbtb7b or another transcription factor (or even CTCF) may be required to recruit or enhance YY1 or PRC2 binding in vLAD border sequences to facilitate repositioning to the lamina. In support of this, a recent study has implicated BTB-POZ domain proteins in the recruitment of PRC2 to chromatin, possibly through interactions with YY1 (Boulay et al., 2012). Intriguingly, in Drosophila melanogaster neurons, competition by BTB-POZ–related proteins for GAGA sites determines the disposition and transcription state of the neural competence factor *Hunchback* (Lehmann et al., 1998; Kohwi et al., 2013). It is therefore tempting to speculate that specific cell type–specific transcription factors can promote or inhibit association with the nuclear lamina through their interactions with chromatin remodelers for the induction of large scale chromatin changes concurrent with either localization to or away from the nuclear periphery. This latter point is important because transcriptional activation appears to disrupt associations with the nuclear lamina (Tumbar et al., 1999; Tumbar and Belmont, 2001; Bubulya and Spector, 2004). Both Zbtb7b and YY1 have been shown to repress and activate genes, adding to the potential for cell type–specific functionality of these proteins in scaffolding to the nuclear lamina.

There are studies in the literature indicating that YY1 may interact with and enhance the activity of HDAC3, thus potentially linking cell type–specific regulation through factors such as Zbtb7b, with a more general model implicating epigenome/genome organizers and specific structural proteins (e.g., LMNA/C and Lap2-β) at the nuclear periphery in positioning chromatin to the nuclear lamina (Figure 2.19; Somech et al., 2005; Sankar et al., 2008; Faulk and Kim, 2009; Atchison, 2014; Luperchio et al., 2014). Interestingly, CTCF has been proposed to interact with YY1 to mediate cell type–specific organization, raising the possibility that CTCF and YY1 act together to target and delimit LAD boundaries (Phillips-Cremins et al., 2013). Numerous studies have implicated the interaction of these two proteins in both nuclear architecture and in gene regulation (Donohoe et al., 2007; Kim, 2008; Nikolaev et al., 2009; Degner et al., 2011; Weth and Renkawitz, 2011; Medvedovic et al., 2013; Wei et al., 2013). Finally, our results also suggest that LMNA/C is important in instructing the peripheral localization of these sequences (Figure 2.9). Two recent studies have implicated LMNA in large scale chromatin organization, but these data did not differentiate between LMNA and LMNC (McCord et al., 2013; Solovei et al., 2013). Our data suggest that either overall levels of LMNA/C are important or, more intriguingly, that LMNC is required for proper genome organization. What is clear from our results is that LMNA is not necessary for recruitment or retention at the nuclear lamina. This suggests, contrary to the commonly held position that there is functional redundancy between LMNA and C, that LMNC may be specifically involved in instructing peripheral positioning. Consistent with this, are studies that “LMNC-only” mice (missing LMNA but with normal LMNC levels) are viable, yet LMNA/C$^{+/-}$ have perturbed differentiation (Fong et al., 2006; Sehgal et al., 2013). Intriguingly, we detect both H3K27me3 and YY1 at the nuclear lamina, especially in YY1-overexpressing cells. Given our overall results, it is tempting to

speculate that YY1 and PRC2 interact with the lamina to facilitate both nuclear positioning and regulation of LADs.

2.5: Materials and Methods

2.5.1: Identification of Lamin Association

Mouse embryonic fibroblasts (FB) were purchased from ATCC (CRL-2752) and cultured according to their establish protocols. *Rag2*$^{-/-}$ proB cells were derived by isolating lineage depleted hematopoietic cells from the bone marrow of B6 (Cg)-*Rag2tm1.1Cgn*/J (JAX catalog # 008449), which harbor a deletion of exon 3 in the coding region of the RAG2 gene. Isolation was followed by *ex-vivo* differentiation and expansion on OP9 stromal cells initially with SCF and IL-7, then IL-7 alone in OptiMEM media with 5% FBS. DamID was performed as described previously (Reddy et al., 2008, Zullo, 2 012). Briefly, self-inactivating retroviral constructs pSMGV Dam-V5 (Dam-Only) and pSMGV Dam-V5-LMNB1 (Dam-LMNB1) were used to generate retrovirus using the Platinum-E packaging cell line (Cell Biolabs RV-101). Supernatants containing viral particles were collected between 48-72 hours post-transfection of the DamID constructs and collection times were pooled. C57BL/6 mouse embryonic fibroblasts were incubated overnight with either Dam-Only or Dam-LMNB1 viral supernatant and 8μg polybrene. *Ex-vivo* expanded Rag2$^{-/-}$ proB cells were spinfected by centrifuging in 24- or 48-well plates at 500g for 2 hours with viral supernatant plus 3ng/mL interleukin-7 (IL-7) and 4μg polybrene at room temperature. Following the spin, the cells were allowed to recover for one hour at in a tissue culture incubator (5% CO_2, 37°C with humidity) before removing the viral supernatants and moving onto OP-9 stromal cells with normal growth medium (OptiMEM, 5% FBS and 3ng/ml IL-7). DamID was performed as previously described

(Vogel, 2007 and Zullo, 2012). Briefly, DNA was isolated from cells expressing Dam or Dam-LMNB1 using Qiagen DNA Mini Kit (51306), precipitated and resuspended to 1μg/μL. 2ug of of this genomic DNA was digested overnight with the restriction enzyme DpnI (NEB R0176L), which cuts at methylated GA^{me}TC. After digestion, double stranded adapters comprising annealed Oligonucleotide *AdRt* (5' –CTA ATA CGA CTC ACT ATA GGG CAG CGT GGT CGC GGC CGA GGA-3') and *AdRb* (5'-TCC TCG GCC G-3') were ligated overnight with the digested DpnI fragments (Roche T4 Ligase 799009) followed by DPNII digestion (NEB R0543S) for 1 hour. This material was amplified in ligation mediated PCR using *AdR_PCR* primer (5'-GGTCGCGGCCGAGGATC-3'), Advantage cDNA polymerase (Clontech, 639105). The resulting material was checked by agarose gel electrophoresis to ensure that specific amplification of methylated DNA fragments occurred and then column purified (Qiagen QIAquick PCR purification kit, 28104). This material was used either for quantitative PCR assays or subjected to array hybridization. For QPCR assays we used the following primer sets: specific to a region of the TCIS insert (*TCIS_ChIP_F1:* AGC TTG GCG TAA TCA TGG TC and *ChIP_R5*: ATT AGG CAC CCC AGG CTT TA), specific to an internal *Igh* LAD region (*J558 1:* AGT GCA GGG CTC ACA GAA AA and *J558 12*: CAG CTC CAT CCC ATG GTT AGA) or to a Lamina-negative region in a LAD (a.k.a. 'Dip') (*chr10:105245772_141bp_F*: AGG GAC AGC CGT GGA GGA GC, *chr10:105245772_141bp_R*: CCG CACC GTC CGG TTC TCA G) (Reddy, 2008). For identification of large genomic regions, Dam-only and Dam-LMNB1 samples were labeled with Cy3 and Cy5 using a random prime strategy, and DamID samples were hybridized to NimbleGen Economy Array mouse whole genome tiling array 3 of 4 (Nimblegen, C7276-00-01 MM8, part of chromosome 9 –part of chromosome 14) using standard protocols. Arrays were scanned using an MS200_2 scanner and probe

intensity $\log_2$ ratios Dam-LMNB1 to Dam-only were obtained using DEVA 1.0.2 software. All experiments were performed two independent times. DamID array signal intensity data was lifted-over to mm9 using the Galaxy converter tool, and then data from replicate arrays were averaged together (Goecks 2010, Blankenberg 2010, and Giardine 2005). DamID data was quantile normalized and smoothed using the preprocessCore R package (Bolstad BM et al), and then segmented via a modified circular binary segmentation using the DNAcopy R package (http://www.bioconductor.org/packages/release/bioc/manuals/DNAcopy/man/DNAcopy.pdf), which is an algorithm for identifying copy number differences which "tests for change-points using a maximal *t*-statistic with a permutation reference distribution to obtain the corresponding *P*-value" (Venkatraman, 2007). A sliding window approach with window size 2kb was used to combine neighboring segments, using in-house perl scripts. Code and accessory scripts are available upon request. Regions identified that were smaller than 25kb were trimmed from total LAD data.

2.5.2: Analysis of LADs and Genomic Features

YY1, CTCF and H3K27me3 ChIP data were tested for statistically significant overlap of LADs in fibroblasts using the GenometriCorr package (Favorov, 2012, Mendenhall, 2010; Peric-Hupkes, 2010; Vella, 2012, Simon, 2013). The GenometriCorr package applies multiple spatial tests of independence including an absolute distance test (i.e. if two elements such as LADs and YY1 sites are a fixed distance from each other), projection test (i.e. testing for significant overlap between these two positions assuming they represent single points points) and the Jaccard test (testing for correlation of these two positions assuming that they are not points, but instead occupy some interval of the genome). In addition to these statistical tests, the intensities of the DamID and ChIP peak data were plotted against bioinformatically determined LAD

border regions (+/-50,000kbp) to generate intensity profiles of the tested binding data for CTCF, YY1, H3K27me3 and LMNB1 using the Genomation R package (Akalin, https://github.com/al2na/genomation). 200 bins were generated over the entire border interval (+/-50,000kbp) and signal intensities of binding data within each bin were averaged and plotted.

2.5.3: Motif Identification

BAC derived fragments DNA tested for repositioning effects were analyzed for known and *de novo* motifs, carried out with MemeSuite and MotifSuite (Bailey, 2009; Claeys, 2012). Specifically, sequences from relocating fragments were analyzed using both MemeSuite (Meme, Multiple Em for Motif Elicitation) using default parameters. Identified motifs were compared to the expected frequency in chromosome 11 background (since the tested fragments were from this chromosome), generated using the MotifSuite background generator tool CreateBackgroundModel. Enrichment of the targeting versus background and non-relocating fragments was determined by utilizing 0.5 threshold parameter. Only motifs that displayed a two-fold enrichment were considered for further evaluation. The identified motifs were then mapped to the fragments with the MAST program (Motif Alignment and Search Tool) in the MEME suite (TL Bailey et al., 1998). We then further confirmed the enrichment using randomly identified sequences in chromosome 11 away from LAD border regions and subjecting these to the same motif mapping via MAST. Only motifs identified as enriched over background on relocating fragments were further examined to identify proteins which may bind to these identified motifs using TomTom motif comparison software (TL Bailey, 2009).

2.5.4: Bacterial Artificial Chromosomes and fragments for positioning analysis

All BACs were obtained from the BACPAC Resource Center at Children's Hospital Oakland Research Institute (Oakland, CA). The listing of BACs with their NCBI clone-IDs and the genes they contain is provided in Figure S1B. DNA fragments for analysis by random integration and switching analysis were created by sheering BAC DNA with a p200 Tip 5-10 times. DNA was then run out on a 0.5% agarose gel and DNA over 25kb was isolated and cloned into a pcDNA3 vector. The pcDNA3 plasmids harboring BAC fragments were then isolated from these clones and screened for BAC DNA insert on an agarose gel. Clones containing fragments from 10-30kb were sent as glycerol stocks to SeqWright Genomic Services (GE Healthcare, Houston Texas) to sequence fragments of BACs from each end. Identified fragments were cloned between the inverted loxP sites of the switch vector, see below. 2.5kb fragments from the DNA fragments of *Ikzf1* fragment F from the vLAD near the gene *Ikzf1* were amplified from the fragments with the following primers: M24_D6_1_w, TGG GAT AGT TCA CAA GAA GCA C, M24_D6_1_C, GTT CAT GCC TAT GGC ACA GC, M24_D6_2_w, ACT TCA TGC TGG GAG ACA GG, M24_D6_2_c, CTC TGC CTG TCC TGA AGC TC, M24_D6_3_w, CCA GTG TGA TGG TGC ATA CC, M24_D6_3_c, GAG GGT GTG TGT GTG TTT GG, M24_D6_4_w, ATC AAC CAA GAG GCC ACA AC, M24_D6_4_c, AGA AGC CTC AGT CCA TCG AG, M24_D6_5_w, TTC CTG GTG CAT CTG TGA AG, M24_D6_5_c, CCT ACG GAG CCA TTT TTC TG, M24_D6_6_w, CAG AAA AAT GGC TCC GTA GG, M24_D6_6_c, ACA GGA GCT GGA GTG GTG AC, M24_D6_7_w, ACT TCA ACC CCC ACT TTT CC, M24_D6_7_c, AGG GAG GCC TTA GAG CTG AC, M24_D6_8_w, GCA GAG TGG AAG CAA GGA AG, M24_D6_8_c, ACC AGG GTG ACC TTG AAC TC, M24_D6_9_w, TAA GGT GGC TGG AGA GAT GG, M24_D6_9_c, TTT TGC AGA TTT CCC CTT TG.

2.5.5: Plasmids used for nuclear compartmentalization analysis

The TCIS vector was constructed by flanking a hygromycin phosphotransferase-thymidine kinase gene (HYTK) with inverted LoxP sites. Subsequently, a 256-repeast *lacO* array was integrated upstream of the LoxP site. DNA fragments (see Bacterial Artificial Chromosomes and fragments for positioning analysis), for recombination, were cloned into a "switch" vector, the original RMCE vector L1HYTK1L (Feng, 1999) was digested with BamHI to remove the CMV promoter and hygromycin/thymidine kinase cassette, the resulting fragment containing the inverted loxP sites was end polished (NEBNext End Repair Module, NEB E6050S). Into this we cloned a HincII/EcoIRCI fragment containing the multiple cloning site from pBlueScript (pBS KS+). Cre Recombinase was introduced by co-transfection with Plasmid 24593, AAV-pgk-cre from Addgene (GenbankID: AY056050). The EGFP-LacI retroviral vector was made by ligating a DraI fragment containing GFP-LacI from p3'ssEGFP-LacI into an HpaI site in pMSCV-puro (Clonetech). YY1-EGFP-LacI fusion proteins were generated by ligating YY1 (Origene plasmid, MC208051 mouse cDNA digested with SalI and PspXI) upstream and in frame with EGFP-LacI in a retroviral vector (p3'ssEGFP-LacI, digested with SalI and PspXI). Zbtb7b-EGFP-LacI fusion protein was generated by ligating Zbtb7b (DNASU plasmid ZBTB7B, pANT7_cGST; clone ID FLH264179.01L), digested with AvrII and PspXI) upstream and in frame with EGFP-LacI in a retroviral vector (p3'ssEGFP-LacI, digested with SalI and PspXI).

2.5.6: Generation and Propagation of C57BL/6 fibroblast cell lines and clones

NIH3T3 derived C57BL/6 murine FB (ATCC: CRL-2752) were transduced with the EGFP-LacI retroviral vector and selected with puromycin (1μg/ml) to generate a line expressing EGFP-LacI. Random integration clones were generated by transfecting the

C57BL/6 EGFP-LacI lines with linearized BACs and hygromycin selectable *lacO* arrays. TCIS cell lines were generated by transfecting C57BL/6 FB with a linearized TCIS construct described above. Cells were selected for hygromycin resistance (500 □g/ml) and clones were isolated and expanded. Single integration clones were screened for by QPCR and transfection with EGFP-LacI retroviral vector to visualize the insert site. Clones 12 and Y had single integrations of the TCIS system at a chromosomal position away from the nuclear lamina, as determined by microscopy and either presence or lack of an overlap in LMNB1 and EGFP-LacI accumulation at the *lacO* insert site. Transfections for TCIS line establishment and random integrations were performed with Fugene 6 (Promega). Site specific recombination was obtained by co-transfection of TCIS clones with DNA fragments cloned into a switch vector and Cre recombinase. Switched cells were then seeded at low density, 10,000 cells per well of a 6 well tissue culture dish and treated with 1μM gancyclovir for 24 hours. TCIS cells require a short treatment with gancyclovir and to be treated at low confluence. Negative gancyclovir selection occurs when the non-switched thymidine kinase gene cassette expresses thymidine kinase, which in turn phosphorylates gancyclovir. Phosphorylated gancyclovir is toxic to the cells, but once released into the media and affect neighboring cells that have successful switched and should be resistant to gancyclovir. Cells resistant to gancyclovir (1 □M) were the for specific recombination in TCIS clones were performed with the Amaxa electroporation system (Lonza Nucleofector 4), to ensure essentially 100% transfection efficiency. Mirus' Ingenio Electroporation Products (Mirus Bio LLC, MIR 50111) were used in combination with the Amaxa nucleofector. All cell lines were maintained in DMEM high with 10% FBS (US Defined Fetal Bovine Serum SH30070.03, Hyclone) in the presence of hygromycin (500μg/ml, Hygromycin B, 30-240-CR 50mg/ml,

Corning/CellGro) and IPTG (1mM) when EGFP-LacI was present. To enable binding of EGFP-LacI, IPTG was removed from the cultures and cells were analyzed after 24 to 36 hours in fresh media. YY1 and Zbtb7b-EGFP-LacI harboring cones were created by transduction of TCIS clones Y and 12 with the retroviral vector and selection with puromycin (1μg/ml).

2.5.7: shRNA knock downs and drug treatments

shLMNA was produced in our laboratory. shRNA mediated 'knock-downs' were carried out by infecting fibroblast clones with freshly produced lentivirus for the specific shRNA. Lentivirus was produced by co-transfecting the desired knock down construct, Delta 8.9 and vsvg vector into HEK293 cells. Virus was added to fibroblast cells at approximately 30-40% confluence and removed after 12-14 hours. New media was added to cells and they were assayed 4 days after infection**.** shRNA constructs were obtained from Sigma Aldridge; shZbtb7b: clone NM_009565.4-2183s21c1, shYY1: clone NM_009537.2-918s1c, shCTCF: clone NM_007794.1-1030s1c1, shEZH2: clone NM_007971.2-421s21c1, shLmnA/C: clone NM_001002011.2-901s21c. , shSuv39H1: clone NM_011514.1-1874s1c1. Off-target controls were shRNA directed against firefly luciferase (CGCTGAGTACTTCGAAATGTC, a gift from the laboratory of Dr. Dan Raben, Johns Hopkins University, School of Medicine, Department of Biological Chemistry). For G9a inhibition, MEFs and TCIS clone lines were treated with 5μM Bix01294 for 24, 48 or 60 hours as, indicated. To reduce H3K27me3, MEFs and TCIS clones were treated with 5μM 3-deazaneplanocin (DZNep) for 24 hours.

2.5.8: Immunofluorescence and 3D DNA-ImmunoFISH

Cells were prepared for IF by platting on sterilized 25mm round coverslips (German borosilicate glass #1.5, 1217N82, Harvard apparatus) in 6 well tissue culture dishes. The nuclear lamina was visualized using an anti-LMNB antibody (sc-6217, Goat IgG, Santa Cruz). IF coupled with DNA in *situ* hybridization on preserved nuclei (3D-DNA-ImmunoFISH) was performed as previously described (Reddy, 2008). DNA hybridization probes were generated by nick translation in the presence of digoxigenin or biotin-conjugated nucleotides (Dig Nick Translation Kit, 11745816910 and Biotin Nick Translation kit, 11745824910, Roche). After probe hybridization an anti-LMNB antibody was used to mark the nuclear lamina. Other antibodies used; Histone H3 (sc-10809, Rabbit IgG, Santa Cruz Biotechnology, 1:1000), GFP (sc-8334, Rabbit IgG,, Santa Cruz Biotechnology, 1:1000), H3K27me3 (39155, Rabbit IgG, Active motif, 1:1000), H3K9me2 (ab1220, Mouse IgG, Abcam, 1:200), H3K9me3 (ab8898, Rabbit IgG, Abcam, 1:500), Zbtb7b (ab20985, Rabbit IgG, Abcam, 1:500), LMNA/C (sc-20681, Rabbit IgG, Santa Cruz Biotechnology, 1:500), LMNA (sc-6214, Goat IgG, Santa Cruz Biotechnology, 1:500), LacI (05-503, Mouse IgG1, Upstate Biologicals, 1:500), YY1 (sc-7341, Mouse IgG1, Santa Cruz Biotechnology, 1:500), CTCF (ab128873, Rabbit IgG, Abcam, 1:500), and β actin (A5441, Mouse IgG1, Sigma, 1:5,000).

2.5.9: Chromatin immunoprecipitation and qPCR

Chromatin immunoprecipitation was performed as previously described (Reddy et al., 2008). Briefly, cells were fixed in 0.5% formaldehyde for 15 minutes and then quenched with saturating amounts of glycine. Fixed cells were lysed in cell lysis buffer containing protease inhibitors [10mM Tris-Cl (pH 8.1) ,10mM NaCl, 0.5% NP-40] for 10 minutes and the nuclei were then pelleted by centrifugation. Nuclei were resuspended in

nuclei lysis buffer containing protease inhibitors [50mM Tris-Cl (pH 8.1), 10mM EDTA and 1%SDS] for at least 10 minutes to generate the chromatin fraction. This chromatin was sonicated to an average length of 600 base pairs and immunoprecipitated with 2.5-5ug of the histone modification specific or control antibody [Antibodies: H3K27me3 (39155, Rabbit IgG , Active motif), H3K9me2 (ab1220, Mouse IgG, Abcam), H3K9me3 (ab8898, Rabbit IgG, Abcam)]. qPCR was carried out using *Igh* primers specific for a region in the center of the locus (distal V gene region) as indicated in Figure S5 and described previously (J558 10, AGT GCA GGG CTC ACA GAA AA and J558 12, CAG CTC CAT CCC ATG GTT AGA) (Reddy, 2008). Primers to test the chromatin state of TCIS insertions were specific to a region of the TCIS insert (TCIS_ChIP_F1, AGC TTG GCG TAA TCA TGG TC and ChIP_R5, ATT AGG CAC CCC AGG CTT TA). Control primers were, Acta2 up, CAG AGG AAT GCA GTG GAA GAG AC and Acta2 down, TCG CTT CCC AAA CAA GGA GC.

2.5.10: Image acquisition and preparation details

All Imaging was done on Zeiss Axiovision inverted fluorescence microscope fitted with an Apotome and Zeiss AxioCam MRm camera. The objective lens used was a Zeiss 100X apochromat oil immersion with a N.A. of 1.5 (Immersol 518, 4449600000000, Zeiss). All immunofluorescence was carried out at room temperature on #1.5 coverslips. 3DImmuno-DNA-FISH studies were either carried out on #1.5 coverslips, or on slides and mounted with #1.5 coverslips. All IF and 3D DNA ImmunoFISH samples were mounted in either VECTASHIELD (H-1000, Vector laboratories) or Slow-Fade Gold (s36937, Life Sciences). Zeiss Axiovision software was used for image acquisition. Images were exported as tiffs to NIH Image J for further analyses. EGFP-LacI/ *lacO* foci were scored as either overlapping with lamin signal (LMNB, peripheral) or not coincident with lamina (central). Images were adjusted using

only brightness and contrast. When comparing expression levels, experiments were done in parallel and imaged at the same exposure times and adjusted in parallel to show differences in protein expression Each experiment was prepared in duplicate and scored as either 'peripheral' or 'not peripheral' by two different people, one of which was blind to the experimental conditions. The data analyzed by these two individuals was compared to ensure accurate measurements. Standard deviation (SD) was then calculated on the average scores from these two observers on two independent experiments for each experiment (n=2, SD refers to SD between experiments). Only data collected by one of the individuals was included for determination of *p*-values to avoid aberrantly inflating the observed 'n'. *p*-values for each experiment and condition were calculated by first converting our scored data into either 'peripheral' (=1) or 'not peripheral' (=0). The distributions of this converted data from a control experiment were compared to the treated/experimental data using an unpaired t-test.

2.5.11: On-line supplemental materials

Scripts for determining LAD domains are provided.

"harr_et_al_rscript_normalization_segmentation_1.txt" provides the commands to use in R to normalize and segment DamID data into LADs.

"harr_et_al_segmentation_consolidate_chrnames_2.pl" and

"harr_et_al_segmentation_refine_3.pl" are used to refine and consolidate LAD domains.

Chapter Three

Tagged Chromosomal Insertion Site (TCIS) System, a method to study lamina associated chromatin

This work is being compiled for an invited contribution to *Intermediate Filament Associated Proteins, Methods in Enzymology*, Elsevier (editors Katherine Wilson and Arnoud Sonnenberg), to be published in January 2016.

3.1: Abstract

Herein we describe a method to study the role of *cis* elements in directing nuclear compartmentalization. The Tagged Chromosomal Insert Site (TCIS) system enables the integration of any DNA fragment into a specific locus to test directed targeting. In addition, this system is designed to assay a targeting event through both cytological and molecular means. Additionally this system can be used to not only study the means of directed targeting to the nuclear periphery but to study the effect of change in environment and the consequences on chromatin state and associated proteins to any nuclear sub-compartment of interest.

3.2: Introduction

The genome appears to be organized on multiple levels such that the spatial location of genes can dictate their interactions with interact with regulatory DNA elements and proteins (Reddy et al., 2008, Zullo et al., 2012, Lin and Murre, 2013, Mao et al., 2011, Luperchio et al., 2014, Wong et al., 2014). The nuclear periphery is enriched in heterochromatin and various studies have demonstrated that some developmental and cell type specific genes dynamically interact with this compartment dependent on cell state and gene activity (Reddy et al., 2008, Finlan et al, 2008, Zullo et al., 2012, Kosak et al, 2002, Szczerbal et al., 2009, Williams et al., 2006, Kumaran and Spector, 2008, Meister et al., 2010, Harr et al., 2015, Zullo et al., 2015). The nuclear periphery is the region of the nucleus that underlies the nuclear envelope. The nuclear envelope is comprise of the inner and outer nuclear membranes (IMN and ONM, respectively), a multitude of resident proteins and in mammalian cells the nuclear lamina, which is composed of the filamentous proteins, B-type and A-type lamins that create a meshwork between the INM and chromatin (Wilson and Berk, 2010). The nuclear lamina has a well-established role in giving structure and stability to the nucleus, maintaining its

spherical shape. New evidence suggests that the nuclear lamina may play a role in maintaining lamina associated domains (LADs) at the nuclear periphery. These large regions of DNA are shown to interact with large regions of the genome (0.1-10 Mb; Guelen et al., 2008; Peric-Hupkes et al., 2010). Additionally, the associations of these regions of the genome with the nuclear periphery are correlated with the presence of specific histone modifications that silence the resident genes (Bian et al., 2013, Towbin et al., 2012, Reddy et al, 2008, Harr et al, 2015). Only recently is evidence emerging to help understand how these large regions interact and are maintained at the nuclear periphery and the role of the nuclear lamina, resident INM proteins, transcription factors and chromatin state in this process.

In this chapter we will describe a new system, the Tagged Chromosomal Insertion site (TCIS). The TCIS system is comprised of a recombination cassette, to allow site specific Cre-mediated heterologous recombination, as well as *lacO* arrays and is integrated in a single copy in a single location in fibroblast cells [Figure 3.1 (Belmont et al., 1999 and Feng et al., 1999)]. Thus, TCIS enables integration of a single copy of any DNA fragment into the genome by directed recombination into the TCIS site and, importantly, aids for visual identification of the disposition of the integrated insert through *lacO*/EGFP-LacI binding. Any genomic element of interest can be specifically recombined into the TCIS system to assay for association with a specific nuclear compartment. Furthermore the *lacO*/LacI component can be utilized for directed recruitment of any protein of interest though discrete tethering to the *lacO* arrays though an X-LacI fusion protein. This system has been used to identify specific DNA elements from variable LADs that are sufficient to target to the nuclear periphery and come in molecular contact with the nuclear lamina in mammalian cells (Harr et al., 2015). Additionally this system has helped us identify proteins involved in this process. While

Figure 3.1: The TCIS System and a flow chart showing the main steps for creating TCIS cell clines and integrating a DNA region of interest. The TCIS system has been designed to allow for site specific integration of any DNA region of interest into the genome. This is accomplished by establishing the TCIS system in a cell line of choice (e.g. murine fibroblasts). A linearized version of the TCIS system is composed of two previously described technologies: the *lacO*/LacI system and recombination mediated cassette exchange (RMCE). This linearized system is integrated into the cell line. The system contains a thymidine kinase hygromycin gene and these integrations will lead to a cell line that is hygromycin resistant. Next, isolate individual clones and screen for cell lines that contain single integrations by qPCR and microscopy (visualizing an EGFP-LacI protein accumulation at the *lacO* arrays). These cell lines can now be used for either recruitment of specific proteins to the *lacO* array through X-EGFP-LacI fusion proteins or by inserting any DNA of interest into the RMCE region of the system. The binding of (X-) EGFP-LacI can be controlled by IPTG addition and removal. In the absence of IPTG, (X-)EGFP-LacI will bind the *lacO* arrays in the TCIS system. To assay sequence directed and/or protein directed reorganization cytological, and molecular means can be carried out.

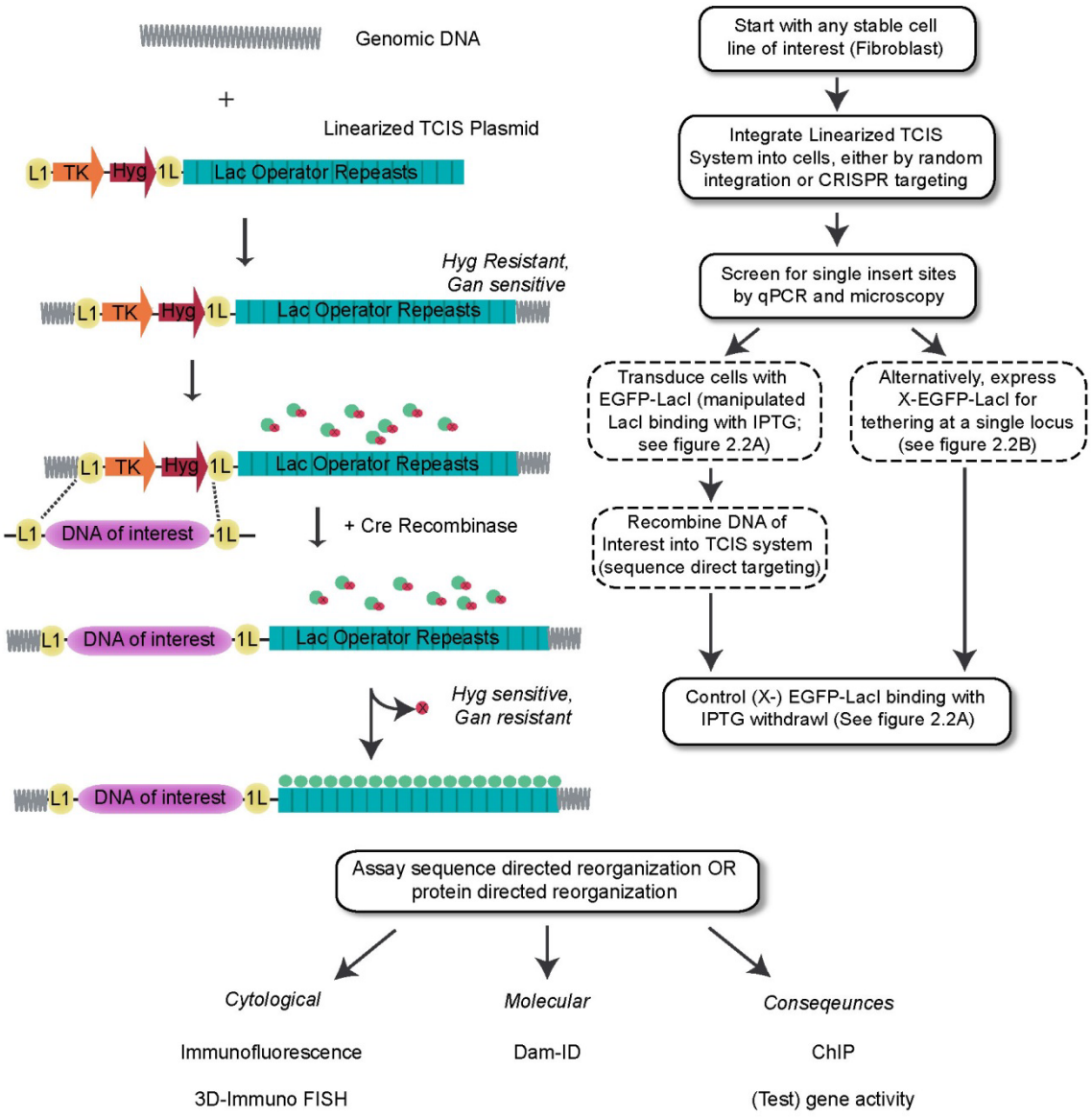


Figure 3.1: The TCIS System and a flow chart showing the main steps for creating TCIS cell clines and integrating a DNA region of interest.

genomic data, from ChIP, DAM-ID and other methods is becoming available to make correlations between these regions, the TCIS system gives us the ability to discretely test the hypothesized associated factors and to assay how chromatin and nuclear periphery associated regions are interacting and in what manner (Figure 3.2). This system allows us to ask questions about LAD organization and formation, *in vivo*- something that genome wide data alone, cannot answer.

3.3: Methods/Results

3.3.1: Design of the TCIS System

3.3.1a: Design and preparation of the TCIS System construct

The system was developed by utilizing two previously described technologies; recombination mediated cassette exchange (RMCE) and the *lacO*/LacI system. RMCE allows for the integration of any DNA sequence of interest and the *lacO*/LacI system aids in visualization of the insert locating with the in the genome and/or site specific tethering (Feng et al, 1999; Belmont et al, 1999). The RMCE section is composed of inverted LoxP sites such that a cassette must always be present. Additionally, the LoxP sites flank a hygromycin phosphotransferase-thymidine kinase gene (HYTK) for both negative and positive selection. Next a 256-repeat *lacO* array is integrated upstream of the LoxP site. This construct is linearized and transfected into mammalian cells to facilitate integration into the genome. One could also introduce this construct by directed targeting using CRISPER technology and directed recombination into a desired location in the genome. When cloning the TCIS system extra care is taken to ensure that the *lacO* arrays do not recombine during amplification of the TCIS system construct in bacteria.

Figure 3.2: TCIS System and recruitment. (A) The TCIS system has been used to investigate the mechanism behind reorganization of chromatin regions to the nuclear periphery. Analysis is done by live cell imaging, immunofluorescence (IF) and 3D immuno-FISH, each obtaining similar results. Determination of interaction with a sub-compartment of interest (e.g. the nuclear periphery) is determined by overlap on IF signals (e.g. EGFP-LacI and anti-Lamin B1). (B) The TCIS system is used to study protein targeting reorganization of chromatin to a sub-compartment of interest (e.g. the nuclear periphery) by tethering a protein of interest to the lacO arrays through EGFP-LacI (X-EGFP-LacI). Top, schematic of reversible IPTG binding, allowing LacI/*lacO* binding upon IPTG withdraw. Bottom, tethering of YY1-EGFP-LacI to the TCIS locus, yiels peripheral targeting of the integrated TCIS system. (C) The TCIS system contains a RMCE region that harbors a thymidine-kinase hygromycin gene. This allow for negative selection after recombination of any DNA of interest. Additionally, PCR can be used to verify the integration of your DNA of interest. Primers are created such that they flank the TCIS system and integrated DNA. A successful integration will yield a gancyclovir resistant culture and a PCR band of predicted size (D).

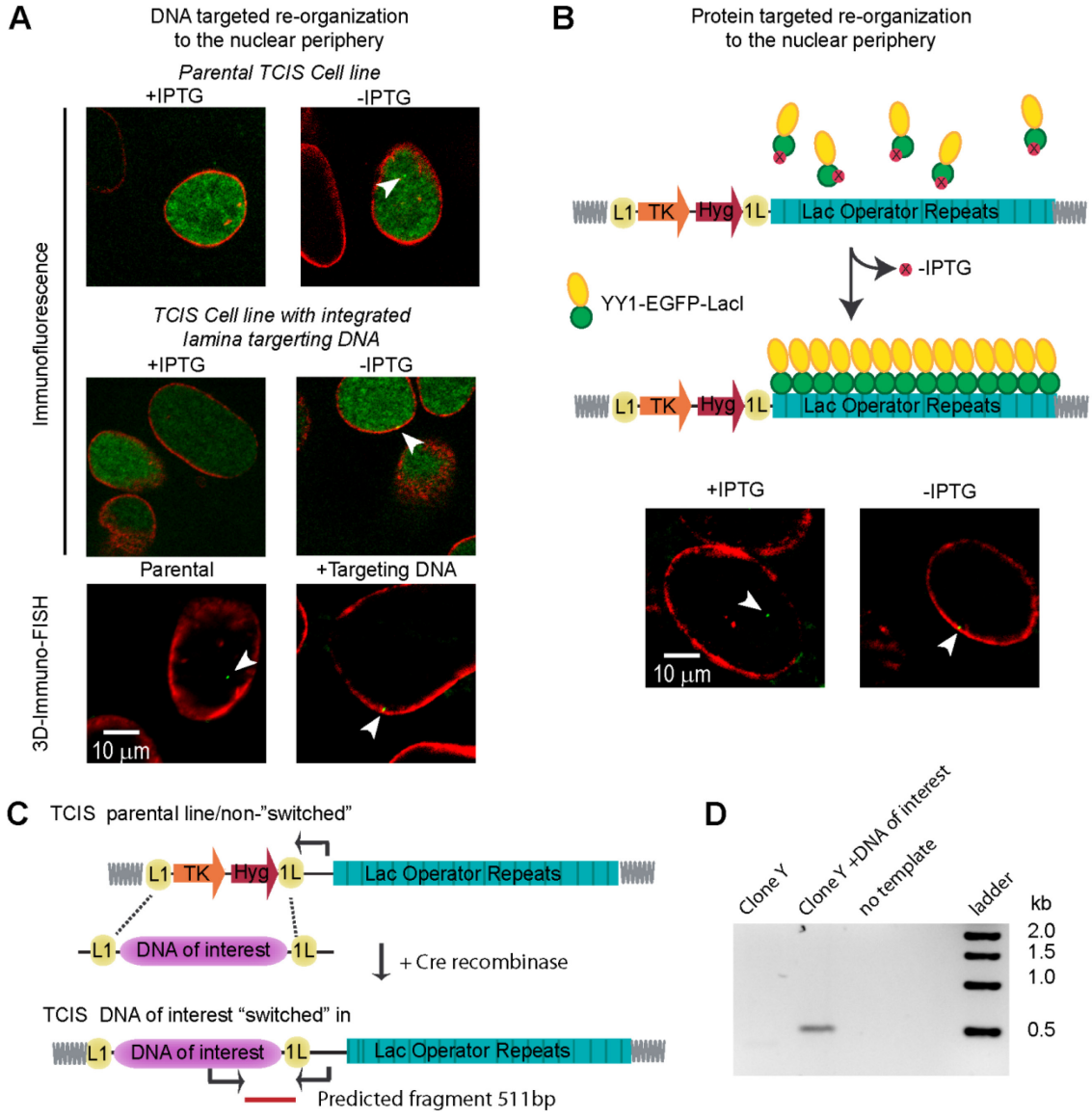


Figure 3.2: TCIS system and recruitment.

The following is a protocol to reduce the occurrence of recombination:

1. Transform electro- or chemically competent Stbl3 (Invitrogen) bacterial strain, or similar, with the above TCIS construct. Stbl3 bacteria, a rag null bacterial line (Stbl3 Competent E. coli, C7373-03, life technologies, genotype: F^- *mcr*B *mrrhsd*S20(r_{B^-},m_{B^-}) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R)*xyl*-5 λ^- *leumtl*-1) can better amplify repetitive or unstable DNA elements (*lacO* array).
2. Plate transformed bacterial into an LB agar plate containing the appropriate selection agent (e.g. Ampicillin).
3. Grow at 30°C overnight (approx. 16 hours). Lowering the incubation temperature will help in reducing recombination events and loss of the full 256 repeat *lacO* array.
4. Pick 24 to 48 colonies (divisible by the number of places in a table top centrifuge, for convenience) and grow in 3ml of LB medium, with the appropriate selection agent, overnight at 30°C. Choosing a lot of colonies will increase the chance of having non-recombined constructs.
5. Prepare DNA from 2ml of the overnight cultures saving some of each on ice or at 4°C for inoculating large cultures after screening.
6. Digest the prepared DNA with the restriction enzyme PvuII, which will yield two bands, a 13.5kb TCIS fragment and the backbone of the full length construct (2.4kb). If recombination has occurred the TCIS fragment containing the *lacO* array will yield multiple bands.
7. Choose 3-6 clones that appear to have not recombined (only two bands are present) to grow large scale cultures overnight. Inoculate 250ml of LB medium containing the selection agent (ampicillin) with the reserved cultures.
8. Grow the large scare cultures overnight at 30°C. The next morning prepare DNA using standard methods (e.g. Alkaline lysis). Digest the DNA with PvuII to verify

the constructs have not recombined. Freeze a small portion (2ml) at -80°C for future use.

3.3.1b: Generation of stable mammalian TCIS clone lines

Generation of stable mammalian clone lines containing the TCIS system allows for the ability to interrogate nuclear organization and structure at discrete loci without effect of position effect or cell line differences. We have created TCIS clones in both murine fibroblast and pro-B cell lines. This system could be adapted to any stable tissue culture line of interest. Additionally the TCIS construct could be engineered to create a mouse line where it is present in every cell.

1. Transfect the linearized TCIS construct (PvuII digest) into your cell line of choice. Transfection with a nonliopsomal transfection agent such as Fugene6 (Promega, E2691) works well.
2. After 48 hours place the cells under Hygromycin B selection (500µg/ml). Concentration of hygromycin will vary depending on the cell type being used. 500µg/ml was determined to work well for TCIS integration into murine fibroblasts. Grow adherent cells so that colonies form, once colonies are visible pick and seed each colony in individual wells of a 24 well plate. For suspension cells, allow the cells to grow under selection for one week and then plate single colonies in a 96 well plate and wait for them to grow up. Continuously grow cultures under hygromycin selection.
3. Verify integration of the TCIS construct by qPCR using specific primers to the system. To determine copy number, compare to a single copy gene.
4. Freeze clone lines that are single copy. These are the parental cell lines.
5. Clones are further selected due to the disposition of the TCIS insert to a nuclear structure of interest. This is done by transiently transfecting or stably transducing

a construct for EGFP-LacI in the cells (see section, *3.3.3a: Design of the EGFP-LacI construct for TCIS visualization).* EGFP-LacI will bind to the *lacO* array in the TCIS system in the absence of IPTG (Isopropyl β-D-1-thiogalactopyranoside), thus marking the location of the TCIS integration in the nucleus, visible by a GFP focus. Once the protein in expressed, in the absence of IPTG, EGFP-LacI will bind the *lacO* arrays rapidly (within 12-24 hours). When IPTG is present in the media (1mM) EGFP-LacI is inhibited from binding *lacO* and no focus is visible. By live cell imaging and Immunofluorescence, in the absence of IPTG, a locus should be visible at the TCIS insert site less than 30% of the time (see section, *3.3.4b. Detection of re-location via immunofluorescence,* for IF protocol). For increased visibility of the foci, 3D-Immuno FISH can be carried out with a probe to the *lacO* array (see section *3.3.4c. Detection of re-location via 3D-ImmunoFISH for quantitative analysis of compartmental association,* for FISH protocol). When comparing IF and 3D-ImmunoFISH studies, the disposition of the TCIS foci is the same for each clone time. When EGFP-LacI is present in the cell line, always grow in the presence of 1mM IPTG. Only withdraw IPTG when conducting an experiment. Cell lines containing EGFP-LacI and the integrated TCIS site should be frozen and stored in liquid nitrogen for future use.

6. Select for stable TCIS containing cell lines that are single copy by qPCR and visualization by immunofluoresnce and EGFP-LacI binding. Additionally choose cell lines in which the integration site an away from the compartment you wish to test. When studying the nuclear periphery, choose clones in which the integration is centrally disposed in the nucleus (and not in contact with the nuclear periphery as marked by anti Lamin B1 staining). If investigating the role of another nuclear sub-compartment ensure your insert is not already in close spatial proximity when investigating targeting to this compartment.

7. Thaw and maintain the parental cell lines of choice at 70-80% confluence. Maintain cell lines under appropriate selection at all times, Hygromycin (500µg/ml) and 1µg/ml puromycin and when EGFP-LacI is being expressed.

These stable cell lines now contain a single integration of the TCIS system and EGPF-LacI. It is recommended to store both a parental cell line with and without the EGFP-LacI protein present. Some experiments may require the use of a different flourophore and/or different tethering protein (X-EGFP-LacI, see section. *2.3.3b: Design of Fusion Proteins utilizing EGFP-LacI*).

3.3.2: Site specific recombination into TCIS cone lines

3.3.2a: Design and creation of DNA to recombine into the TCIS site

Any segment of DNA of interest can be recombined into the stably integrated TCIS system in established TCIS clones. The DNA of interest is easily obtained by purchasing readily available Bacterial Artificial Chromosomes (BACs, BACPAC Children's Hospital Oakland Research Institute in Oakland, California, USA) to a region of interest or by amplification from gDNA by specific primers to the region of interest. DNA of interest is cloned into a “switch” vector in which the DNA is flanked by inverted loxP sites. This vector is created my restriction digest (BamHI) of the original RMCE vector L1HYTK1L (Feng, 1999) to remove the CMV promoter and hygromycin/thymidine kinase cassette. We then cloned into the resulting end polished BamHI, LoxP containing fragment, a HincII/EcoRI fragment containing the multiple cloning site from pBlueScript (pBS KS+). The multiple cloning site allows for easy integration of any DNA of interest into the “switch” vector.

3.3.2b: Recombination of DNA into the TCIS site

After creation of a "switch" vector containing your DNA of interest, co-transfection of the prepared "switch" vector and Cre- recombinase expression vector into the stable TCIS clone line, will lead to the DNA of interest becoming integrated into the TCIS site by site specific recombination. The following protocol is recommended for highly efficient transformation (90-100%) and successful integration of your DNA of interest.

1. Defrost parental TCIS clone lines and grow to 70-80% confluence. Make sure to grow cells under Hygromycin selection (500µg/ml).
2. Transiently transfect cells by electroporation with the Amaxa electroporation system (Lonza, Nucleofector 4) using Mirus' Ingenio Electroporation Products (Mirus Bio LLC, MIR 50111 or Lonza electroporation products). To Harvest cells, remove media and wash once with PBS. Add 0.05% Trypsin/EDTA to remove adherent cells form the dish. Quench with an equal amount of media and count cells. Each electroporation reaction will be for 10^6 cells.
3. Centrifuge cells at 90 g, room temperature (25°C) for 10 minutes to pellet cells. Re-suspend cell pellet in 110µl/ reaction of room temperature Mirus' Ingenio electroporation solution.
4. Add 110µl cells to a 1.5ml tube containing 1-5µg highly purified plasmid DNA (in maximum 5µl). For recombination use 1:1, 2.5µg Cre-recombinase (Plasmid 24593, AAV-pgk-cre from Addgene, GenbankID: AY056050) and 2.5µg "switch" vector harboring DNA of interest. Transfer the cells and DNA mixture to a cuvette and ensure sample is at the bottom of the cuvette and no bubbles are present.
5. Electroporate cells with appropriate Amaxa program (U-30 for Murine fibroblasts). Immediately transfer cells from the cuvette to a 1.5ml tube containing 500ul, 37°C RPMI media and incubate cells at 37°C for 15 minutes. The incubation in calcium free media will allow cells to recover after electroporation and leads to higher

survival of the cells. Process no more than 6 samples at a time, to avoid storing cells in electroporation solution for more than 15 minutes.

6. Transfer cells to one well of a 6 well dish containing pre-warmed (37°C) growth media (DMEM high, 10% FBS, pen/strep and glutamine), and place in a 37°C/5% CO_2 incubator. Do not disturb cells for 24 hours. IMPORTAINT: At this point, no longer maintain the cells with hygromycin selection. Upon successful recombination the hygromycin phosphotransferase-thymidine kinase gene is no longer present. We find that use of Amaxa transformation yield nearly 100% transient transfection efficiency with minimal cell death. Within 7 days of transfection all transient DNA in absent from the cells. This is integral to studying single TCIS specific DNA recombination; you want to ensure that DNA other than at the desired location is not present transiently or by random integration into the genome.
7. After growing cells for 24-48 hours, seed at 10,000 cells per well of a 6 well dish and add gancyclovir (1μM) to DMEM high media (10%FBS, Pen/step and glutamine). Incubate at 37°C/5% CO_2 incubator for 24 hours.
8. Remove media after 24 hours and replace with DMEM high (10% FBS, pen/strep and glutamine). Culture cells to maintain 10-40% confluence for the first week, changing the media every 24 hours. Cells that have successfully recombined and taken up the DNA fragment of interest, will no longer contain the hygromycin phosphotransferase-thymidine kinase gene. In the presence of gancyclovir, these cells are not affected. If recombination was not successful and the hygromycin phosphotransferase-thymidine kinase gene is still present, the cells will phosphorylate gancyclovir by the expressed thymidine kinase. Phosphorylated gancyclovir is toxic to the cells and once released into the media will affect neighboring cells that have successfully switched and should be resistant to

gancyclovir, so cells are maintained at a low confluence and the media is changed regularly until a stable line is produced.

9. Cells resistant to gancyclovir (1μM) are then expanded for nuclear positioning analysis. These cell lines should be frozen and stored in liquid nitrogen for future use.

3.3.3: LacI/lacO recruitment

Controllable recruitment of EGFP-LacI to the *lacO* arrays of the TCIS system is used to visualize the stable integration site. Accumulation of EGFP-LacI (after IPTG withdrawal) at the 256 *lacO* array, leads to visualization of the TCIS system by way of a small foci, visible by live cell imaging, Immunofluorescence or FISH. Additionally a fusion protein can be designed such that a protein of interest is tethered to the *lacO* array through the LacI and is visible by the presence of EGFP.

3.3.3a: Design of the EGFP-LacI construct for TCIS visualization

The EGFP-LacI retroviral vector was made by ligating a DraI restriction digested fragment containing GFP-LacI from p3'ssEGFP-LacI into an HpaI site in pMSCV-puro (Clonetech). This vector is transiently transfected into a viral packaging line (PlatE, Platinum-E, retroviral packaging Cell line, Cell biolabs, Inc), 24 hours after transient transfection, media is replaced with DMEM high, 10% FBS, pen/step, L-glutamine. Media is collected and replaced at, 36, 48 and 60 hours after transfection. The EGFP-LacI packaged retrovirus containing media can be snap frozen on liquid nitrogen and stored at -80°C for later use.

Parental TCIS cell lines are infected with the EGFP-LacI virus for 24, in the presence of 0.5μg/ml polybrene. After 24 hours incubation the virus is removed and

replaced with DMEM high (10%FBS, pen/strep and glutamine) and 1µg/ml puromycin is added to the cells to select for presence of EGFP-LacI.

3.3.3b: Design of Fusion Proteins utilizing EGFP-LacI

The *lacO* array of the TCIS system can be used to discretely tether any protein of interest to a discrete mammalian locus. By creating a protein X-EGFP-LacI fusion protein, one can control the binding of protein X at the TCIS locus, in the absence of presence of IPTG. X-EGFP-LacI fusion proteins are generated by ligating the cDNA for a protein of interest (lacking a stop codon) upstream and in frame with EGFP-LacI in a retroviral vector (p3'ssEGFP-LacI).

3.3.4: Assaying Recruitment/ re-organization

After successful integration of a fragment of DNA of interest or expression of a fusion X-EGFP-LacI protein in a TCIS cell line one can start to assay targeting to a specific nuclear compartment. An example is to assay for the association of either the targeting fragment or tethered protein to the nuclear periphery. The nuclear periphery is regularly demarcated by the lamina network that underlies the inner nuclear membrane. Directed targeting can be assayed by both cytological and molecular means.

3.3.4a: Targeting a DNA fragment or discreetly tethered protein to a nuclear compartment

1. Getting cells ready

A. Thaw cells and grow to 70-80% confluence. For investigation of targeting sequences thaw cell lines containing DNA of interest, a non-targeting fragment and the parental cell line all harboring EGFP-LacI. For X-EGFP-LacI fusion tethering studies, thaw TCIS cell lines containing EGFP-LacI and X-EGFP-LacI.

Grow cells in selection media for the insert (or lack of) and for the EGFP-LacI and all under 1mM IPTG. X-EGFP-LacI fusion harboring cultures should be kept under hygromycin (500µg/ml) selection at all times. Cell lines containing recombined DNA of interest are no longer resistant to hygromycin and should no longer be kept under selection.

B. When cell reach 70-80% confluence, plate onto coverslips, slides or a 6 well dish depending on the experiment (see below).

2. Re-organization

A. Once the cultures on either coverslips/slide or coverslips reach 50% confluence, remove the media and replace with media plus 1mM IPTG or media alone. Do not include selection reagents at this time. For DNA targeting experiments you should have a control line of cells containing the parental TCIS insert, and those containing the DNA of interest (experimental line). All of these are minus IPTG. For tethering fusion proteins you should have two sets of the following; cells harboring EGFP-LacI, and X-GFP-LacI. One set contains IPTG and the other is absent of IPTG. The X-GFP-LacI cells minus IPTG are the experimental cell line.

B. Grow the cells for 24-48 hours.

C. Analyze the cells as outlined below.

3.3.4b. Detection of re-location via immunofluorescence

1. Getting cells ready

Grow Adherent cells on coverslips and for suspension cells attach to coverslips by coating with poly-L-lysine. Never allow cells to dry out during the following procedures. 25mm round coverslips fit well in a 6 well dish and 12mm coverslips in a 24 well plate.

2. Fixation

A. Remove media from cells and add 4% formaldehyde in 1x PBS to cells for 10-15 minutes. For murine fibroblasts we use 10 minutes. Times are empirically determined for each cell type.

B. Rinse three times in 1x PBS (2-3 minutes each)

3. Permeabilization

A. Incubate coverslips in 0.5% Triton X-100 in 1x PBS for 10-20 minutes.

B. Rinse three times in 1x PBS (5 minutes each).

4. Blocking and primary antibody incubation

A. Block slides in 4% BSA in 1x PBS for 1-2 hours at room temperature in a humid environment. A humid chamber can be created by soaking a paper towel in water and placing this in the covered container in which you are storing your coverslips. Place blocking solution on a piece of parafilm. Place coverslip, cells facing down, onto the bubble of blocking media. Use approximately 100µl for 25mm round coverslips and 50µl for 12mm coverslips.

B. Dilute primary antibodies in blocking solution. The dilution used will depend on the antibody. Start with the recommended concentration for immunofluorescence. The recommended concentrations for western blot and ChIP are not necessarily ideal for IF. The first time an experiment is done, test several dilutions and use the concentration that yields the best signal to noise ratio.

C. On a new piece of parafilm place the diluted antibodies and transfer the coverslips from the blocking solution onto the antibody solution (cells facing down, as above). If the coverslips are difficult to remove, take 100µl blocking solution and pipette under the edge of the coverslip, this will help loosen them up. Incubate in a humid chamber at room temperature for at least one hour, or overnight at 4°C.

D. Wash coverslips three times in 1x PBS for 10 minutes each. Gently remove coverslips from the parafilm and return (cells facing up), to a 6 well dish. A more

stringent wash can be done by adding 0.1% Triton- X 100 to the 1x PBS used for washing.

5. Secondary Antibody detection and mounting

A. Dilute secondary antibodies in the blocking solution. Incubate cells in antibodies as done above.

B. Incubate in a humid chamber for 1-2 hours at room temperature.

C. Gently return over slips to the culture dish (cells facing up) and wash three times in 1x PBS for 10 minutes each.

D. If desired, stain cells in DAPI or Hoechst (1/10,000 dilution in 1x PBS, 10µg/ml stock solution) for 30 seconds to 1 minute. Rinse once with 1x PBS. We do not recommend using a mounting media containing DAPI. Our recommended short incubation time with Hoechst allows for visualization of fine details that can be swamped out with over staining.

E. Prepare slides by adding 25ul VECTASHIELD (H-1000, Vector laboratories) or Slow-Fade Gold (s36937, Life Sciences) mounting media. Gently dry coverslip by taping the edge against a dry paper towel and invert the cover slip (cells down) onto the mounting media. Blot excess mounting media from the slide by very gently pressing with a paper towel. Seal the coverslip with nail polish.

Allow slides to dry for a few hours to overnight before imaging. This incubation time will allow for the mounting media, which contains an anti-fade solution to fully penetrate the sample and for the nail polish to dry. Slides can be stored at 4°C for a few weeks.

3.3.4c. Detection of re-location via 3D-ImmunoFISH for quantitative analysis of compartmental association.

3D immuno-FISH allows for visualization of the TCIS insert site and endogenous proteins or by expression of X-EGFP-LacI. This allows for quantitative analysis of the TCIS disposition within the nucleus with respect to the compartment or protein of interest. In the absence of IPTG, the TCIS insert cannot be visualized by immunofluoresnce due to the lack of accumulation if (X-)EGFP-LacI at the TCIS insert site. To image these control cells, FISH must be done. The protocol presented here is an adaption from Solovei et al. (2002) and Reddy et al. (2008), see these protocols for more detailed information.

1. 3D Immuno FISH Day 1: preparing slides and making probes

A. Prepare cells. Plate cells onto UV sterilized slides in a larger dish. For adherent cells: puddle 250,000 cells in 250µl media (a 25mm diameter spot) onto slides and let sit for 5-10 minutes. Flood dish with media. Allows cells to fully adhere by incubating at 37°C/5% CO_2 for a few hours to overnight. For suspension cells: Puddle 0.5-1 x 10^6 cells in a 25mm diameter spot on a UV sterilized poly-L-lysine coated slide. Let cells sit for 5-10 minutes and flood dish with media.

B. Fix cells. To ensure that fixation does not affect nuclear morphology, stain the cells on one slide with a viable nuclear stain like Hoechst, measure nuclear diameter and look at general morphology. Keep this in mind to ensure that nuclear volume and morphology is maintained after completing the FISH protocol.

I. Fix cells in 4% paraformaldehyde in 1xPBS for 10-20 minutes. Fixative can be added directly to the tissue culture dish (into the media) or done in a coplin jar. Fibroblasts are fixed for 15 minutes.

II. Wash 3 times in 1x PBS (5 minutes each).

C. First Permeabilization: Make a fresh solution of 0.5% Triton X-100 and 0.5% Saponin in 1x PBS. Incubate slides for 10-20 minutes and then rinse three times for 5 minutes each in 1x PBS.

D. HCL treatment: Make 0.1N HCl and 20% glycerol in 1x PBS (w/v). Incubate slides in 0.1N HCl for 10 minutes. Immediately flick excess HCl from slides and place into 20% glycerol solution. Incubate in glycerol/1xPBS for at least 1 hour. Continue with freeze thaw or store overnight at 4°C.

E. Make FISH probes according to standard nick translation protocols. We use the direct labeling or dig- bio- labeling kits available from Roche (Catalog # 11745816910 and 11745824910). To detect the TCIS insert site, label the plasmid DNA previously prepared. When making probe ensure that, the majority of the probe is between 200 and 700 bp in size.

2. 3D Immuno FISH Day 2: Freeze thaws Permeabilization and probe hybridization

A. Freeze/Thaw Permeabilization

I. Continue from above, or remove slides from 4°C.

II. Fill a Styrofoam container with Liquid nitrogen. Ensure there is enough liquid nitrogen to fully submerge a glass slide. With a large pair of forceps pick up one slide and submerge into the liquid nitrogen. Keep slide in liquid nitrogen until fully frozen. You will hear a “click” and/or bubbling will stop.

III. Place the slide on a plastic grid to fully thaw.

IV. Repeat for every slide. Repeat 4 times for each slide, re-submerging in glycerol every other freeze, to ensure that the slide does not dry out.

V. Place slides at -80 degrees in a slide mailer if not using immediately. Slides can be frozen indefinitely.

B. Prepare probe

I. Take 0.3ug of labeled DNA (from step *2.3.4c 1.E)* and add Cot-1 (3ug/sample), Placental (6ug/sample), and sonicated salmon sperm (9ug/sample) DNA. These DNAs are the only blocking agents present during the hybridization procedure.

II. Precipitate DNAs for 30 min to 1 hour at -80°C.

III. Resuspend DNA in hybridization buffer (50% deionized formamide in 2xSSC with 10% dextran sulfate) by vortexing at 4°C for at least 1 hour.

IV. Denature for 5 minutes at 80-94°C.

V. Pre-anneal for 10 minutes- 2 hours at 37-42°C.

C. RNase treatment

I. Rinse slides in 1x PBS three times.

II. Prepare 50-100ug/ml RNase in 2x SSC. Place 200µl RNase solution on the slide and gently cover cells on the slide with a 24x 66mm glass coverslip. Incubate for 1 hour at room temperature in a humid chamber.

III. Rise slides in 2x SSC and leave in 2x SSC until hybridization.

D. Denaturation/hybridization

All hybridization steps should be carried out in a chemical hood while wearing appropriate personal protective equipment. Formamide is highly corrosive and acts as a teratogen.

I. Warm up slide warmer to 37°C.

II. Prepare 50ml deionized formamide in 2xSSC, pH 7.0 and 50ml formamide in 2x SSC, pH 7.0. If planning to hybridize more than 4 slides, prepare two batches of both solutions.

III. Place solutions in coplin jars and set water bath to 75-80°C. The temperature of the solution should be at 75°C- this will yield a hybridization temperature of 72°C when the temperature drops with addition of the slide.

IV. When the solutions are 75°C, take one slide and place it into the 70% formamide solution for exactly three minutes.

V. Immediately transfer the slide into the 50% formamide solution for exactly one minute.

VI. Place 10µl hybridization cocktail, containing probe onto a 18x18mm coverslip on the slide warmer.

VII. Remove the slide from 50% formamide solution and flick of excess liquid. Quickly place the slide (cell slide down) onto the coverslip. Flip-over and seal with rubber cement. Allow rubber cement to set up on the slide warmer.

VIII. Transfer the slides to humid chamber and incubate at 37°C overnight to several days.

E. Detection of FISH probes and Immunofluorescence

I. Remove rubber cement from slides and coverslips and place slides in 2x SSC. Dipping the slide and coverslip into 2xSSC will help in removing the slide.

II. Wash slides three times in 50% formamide in 2x SSC at 42°C for seven minutes each.

III. Wash slides three times, seven minutes each in 0.1-0.5 x SSC at 63°C.

IV. Block cells in 4% BSA in 2xSSC for 30 minutes to 1 hour at room temperature in a humid chamber. Place block solution on the slide and hold in place using a coverslip.

V. Add primary antibody at suggested concentration in blocking solution. Incubate for 1 hour at room temperature or overnight at 4°C.

VI. Wash slides three times in 2xSSC with 0.1% Triton X-100 at 37°C for 5 minutes each.

VII. Add secondary antibodies for detection. For bio or dig labeled probe, a secondary for streptavidin or digoxigenin, respectively, and coupled to a flourophore is used. For any other antibodies used to mark specific nuclear proteins and or compartments, use a secondary antibody against the species of your primary antibody. Dilute antibodies at appropriate concentrations in blocking solution. Incubate for 1 hour at room temperature.

VIII. Wash slides three times in 2xSSC with 0.1% Triton X-100 at 37°C for 5 minutes each and place in 2x SSC.

IX. If desired, stain slides in Hoechst (1:10,000 dilution from a 10ug/ml stock solution) in 2x SSC, for 30 seconds to 1 minute. Place slides in 2x SSC until mounting.

X. Mount slides by flicking the slide to remove as much excess 2xSSC as possible. Then add a small amount (100µl) of an anti-fade agent (Vectashield or Slowfade gold) drop wise to the slide. Cover with a large coverslip (#1.5) and gently blot to remove excess mounting agent. Use nail polish to seal the edges and hold the coverslip in place.

XI. Store slides, flat and in the dark at 4°C until used for imaging.

3.3.4d: Determination of Molecular contact-Dam-ID

TCIS system was designed to visually track the targeting of a single locus within the nucleus. The ability to visualize the TCIS system through the binding of EGFP-LacI/*lacO* is easily assayed by microscopy but is limited by yielding only cytological data. The protocol DNA Adenine Methyltransferase Identification (or Dam-ID) has been adapted to assay molecular contact between DNA and the nuclear lamina or any nuclear

proteins of interest (INM proteins, L2β exct.) This protocol was originally published by Griel et al. (2006) and adapted for mammalian cells as described in Vogel et al. (2006), Reddy et al. (2008) and Zullo et al. (2012). Herein we describe the protocol for marking interaction of DNA with the filamentous lamin proteins underlying the nuclear periphery.

1. Creation of DamID Virus. DamID constructs should be stably expressed in your cell line of interest at a very low concentration. Self-inactivating retroviral constructs pSMGV Dam-V5 (Dam-Only) and pSMGV Dam-V5-LMNB1 (Dam-LMNB1) were used to generate retrovirus (see *3.3.3a: Design of the EGFP-LacI construct for TCIS visualization,* for Virus production protocol). The Dam-Only construct is critical to determine base background levels of DNA adenine methyl transferase activity within the nucleus and is your control throughout the experiment.
2. TCIS clone cells are defrosted and seeded to be at 40-50% confluence and were incubated overnight with either Dam-Only or Dam-LMNB1 viral supernatant and 8µg polybrene.
3. Cells are grown for 48-60 hours while harboring the DamID constructs.
4. Cells are then harvested for DNA isolation using Qiagen DNA Mini Kit (51306) and DNA is resuspend at 1µg/µL.
5. 2µg of the genomic DNA for each condition is then digested overnight with the restriction enzyme DpnI (NEB R0176L), which cuts at methylated GA^{me}TC. This will cut at any site in which the Dam fusion protein has come in contact and marked GATC with a methylated adenine.
6. After digestion, double stranded adapters comprising annealed Oligonucleotide *AdRt* (5' –CTA ATA CGA CTC ACT ATA GGG CAG CGT GGT CGC GGC CGA GGA-3') and *AdRb* (5'-TCC TCG GCC G-3') are ligated overnight with the digested DpnI fragments (Roche T4 Ligase 799009).

7. The ligated material is then digested by DpnII (NEB R0543S) for 1 hour. This digestion step ensures that any non-methylated GATC regions are not included in the analysis.
8. The resulting material is then amplified by ligation mediated PCR using *AdR_PCR* primer (5'-GGTCGCGGCCGAGGATC-3') and Advantage cDNA polymerase (Clontech, 639105).
9. The resulting amplified material is then checked to ensure that specific amplification of methylated DNA fragments occurred, by running a small sample on agarose gel electrophoresis. The amplified material should appear as a broad smear of equal intensity for all samples.
10. Next, Column purify the remaining amplified material (Qiagen QIAquick PCR purification kit, 28104).

The column purified material can now be used for quantitative PCR assays. For testing of molecular contact of either DNA targeted or X-EGFP-lacI fusion proteins directed nuclear peripheral association in TCIS fibroblast, we carry out qPCR analysis using a primer set specific for the TCIS insert (*TCIS_ChIP_F1:* AGC TTG GCG TAA TCA TGG TC and *ChIP_R5*: ATT AGG CAC CCC AGG CTT TA), specific to an internal *Igh* LAD region (*J558 1:* AGT GCA GGG CTC ACA GAA AA and *J558 12*: CAG CTC CAT CCC ATG GTT AGA) or to a Lamina-negative region in a LAD (a.k.a. 'Dip') (*chr10:105245772_141bp_F*: AGG GAC AGC CGT GGA GGA GC, *chr10:105245772_141bp_R*: CCG CACC GTC CGG TTC TCA G) (Reddy, 2008). The primers to an internal *IgH* Lamina associated regions are a positive control for lamina association, while the primers to the "dip" region are a negative control.

3.3.4e: Consequences of relocation: determination of histone modifications due to re-organization-ChIP

While the TCIS system enables us to study directed targeting of a specific region of chromatin by cytological and molecular means, we can expand upon this study by examining the consequences of nuclear re-organization of a discrete locus. The TCIS locus can be targeted to any region of interest in the nucleus (e.g. the nuclear lamina) and then one can father investigate the changes in chromatin state before and after re-location by Chromatin Immunoprecipitation (ChIP). The following ChIP protocol is adapted from Reddy at al. (2008).

1. Prepare cells. Seed cells 1-2 days before fixation, so that the cells will be 70-80% confluent on the day of fixation. For each ChIP, process between 20-100 x10^6 cells, depending on downstream step requirements.
2. Once cells reach confluence, fix in 0.5% formaldehyde for 15 minutes. Add formaldehyde directly to the tissue culture dish and rock gently.
3. Immediately after fixation quench with saturating amounts of glycine (0.125mM).

   IMPORTAINT: For all following steps keep cells, and reagents on ice. Supplement all regents with fresh protease inhibitor.

3. Rinse cells with cold 1xPBS.
4. Add 10ml 1xPBS scrape cells from dish using a flat cell scrapper. Transfer cells to a chilled 50ml tube.
5. Rinse dish one more time with 5 ml 1x PBS to collect remaining cells and transfer to 50 ml tube.
6. Once all cells have been collected, Centrifuge at 2,500g for 10 minutes to pellet the cells.
7. Decant the supernatant and lyse cells in cell lysis buffer containing protease inhibitors [10mM Tris-Cl (pH 8.1) ,10mM NaCl, 0.5% NP-40] for 10 minutes at 4°C.

8. Next pellet the nuclei by centrifugation, 10 min at 4°C.
9. Decant the supernatant and resuspend nuclei in nuclei lysis buffer containing protease inhibitors [50mM Tris-Cl (pH 8.1), 10mM EDTA and 1% SDS] for at least 10 minutes to release the chromatin.
10. Sonicate chromatin to an average length of 600 base pairs, by probe sonication or with a Biorupter (Diagenode). We find that use of the Biorupter, gives optimal results.
11. Dilute sonicated DNA to 1-5 10^6 cell/ml in IP dilution buffer (50mM Tris-HCL, pH 7.4, 150mM NaCl, 1% (v/v) igepal, 1mM EDTA, pH 8 with protease inhibitors) and aliquot in 1.5ml tubes. These samples can be frozen on dry ice and stored at -80°C or used directly for immunoprecipitation. Note: store samples in 1mL aliquots and save a few vials at 5-20% this volume to use in a later step as your input sample.
12. Add 2.5-5ug of the histone modification specific or control antibody (e.g. H3K27me3 (39155, Rabbit IgG, Active motif), H3K9me2 (ab1220, Mouse IgG, Abcam), H3K9me3 (ab8898, Rabbit IgG, Abcam)) to each 1.5 ml tube of chromatin. Incubate overnight at 4°C.
13. Add magnetic capture beads (e.g. Protein G magnetic dynabeads, Invitrogen 100.04D) to the chromatin and antibody mix and incubate with constant rotation for 1-2 hours at 4°C. Do not exceed 2 hours of incubation time. After the incubation, your chromatin is now bound to your anti-body of interest and in turn bound to the magnetic beads though Protein G. At this step include an input samples. This is used to determine percent of input during final qPCR analysis. Input can be 5-20% of your total chromatin sample.
14. Pellet beads using magnetic capture and carefully remove the supernatant that contains the unbound antibody and chromatin fraction. Wash the magnetic beads bound to the antibody/DNA complex for 5 minutes each with 1mL of the following wash buffers with 1x protease inhibitor: low salt wash buffer (1% Triton X-100, 2mM

EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), high salt wash buffer (1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), LiCl wash buffer (0.25M LiCl, 0.5% IGEPAL, 1mM EDTA, 10mM Tris, pH 8.1). Then wash once with 1x 10mM Tris, EDTA.

IMPORTAINT: Be careful and consistent with pipetting during washes. To quantitate by qPCR after ChIP you do not assay for DNA concentration but test against input samples and compare relative amounts of your target region pulled down by the tested antibody all as compared to an IgG control.

15. Elude DNA from beads by incubating in elution buffer (50mM $NaHCO_3$, 1% (w/v) SDS), at room temperature with constant rotation for 30 minutes, followed by a 10 minute incubation at 37°C. Save 10% of your elute sample or boil beads (after elution) with 2x SDS page loading buffer for western analysis.
16. Reverse cross-link the eluted DNA/antibody complex by adding 10% of the volume, NaCl (5M), incubate overnight at 65°C.
17. After reverse cross-link, RNAse treat samples and column purify DNA.

Next qPCR is carried out on your eluted DNA. For investigation of the chromatin state of the TCIS locus before and after lamina association we use primers to a specific region of the TCIS insert (TCIS_ChIP_F1, AGC TTG GCG TAA TCA TGG TC and ChIP_R5, ATT AGG CAC CCC AGG CTT TA). Primers should be designed as both positive and negative controls for each histone modification assayed.

3.4: Concluding remarks

Mounting evidence supports that the nucleus is organized into discrete compartments that regulate gene activity and function. In this chapter we describe a method to assay the directed re-organization of chromatin to any nuclear compartment/body of interest. This system has been used to successfully identify DNA

sequences from variable lamina associated domains that have the ability to target to the nuclear lamina in single copies. Furthermore directed tethering of a protein, YY1, identified in the process of re-organizing chromatin to the nuclear lamina can be visualized and quantitated by both cytological and molecular methods (Harr et al., 2015). Additionally this system has allowed us to examine the consequences of association of discrete loci with the nuclear lamina and periphery. Importantly this system can be expanded to examine any nuclear compartment of interest with the discovery of new targets and regions of interest, through either discovery of new targeting DNA cis elements and/or specific proteins that when tethers to the *lacO* arrays and lead to specialized association with a compartment of interest.

Chapter Four

Concluding Remarks

This dissertation presents a novel method for understanding the structural and functional relationship between epigenetic modifications and nuclear architecture. It has been observed that the dynamic organization of the nucleus contributes to regulation of gene expression in both normal cell state, and throughout development and loss of organization has been implicated in disease. How the overall three-dimensional structure of the nucleus plays a role in these processes is only beginning to become understood. With the advent of genome wide methods to profile the molecular interactions of nuclear sub-domains and protein interactions, there will be a lot of information from a variety of cell lines at different time points in development, that we must make sense of. What is important to note is that these genome wide studies are all done on a population basis and may not truly represent the exact state in a single cell. To understand how structural population studies apply to functionality in the cell and the mechanisms behind the process, we need to ask questions that directly probe chromatin state and effects on nuclear organization in a testable/controllable way.

Genome-wide LAD data from two different cell types has les up to identify vLAD regions that contain developmentally regulated genes. We hypothesize that these vLAD regions act as a level of regulation over the genes expression. We use the Tagged Chromosomal Insertion Site (TCIS) system to determine structure-function relationships of the vLAD DNA sequences. The TCIS system makes it possible to have site specific recombination of any DNA of interest at a specific locus in the murine genome. This allows for investigation of cis targeting elements without the pitfalls of random integration studies that are susceptible to positon effect and are done in multiple copies that can confound the results. Additionally we can recruit chromatin modifying and structural proteins of interest to the same site through *lacO* arrays. The TCIS system makes it possible to determine the mechanism behind the structural and functional relationships that are emerging.

The role of the nuclear periphery as a regulatory compartment to control gene regulation is only truly starting to become understood. By integrating genome-wide data (Dam-ID) in multiple cell types, we have identified regions of the genome that are lamina associated and vary depending on cell type. We have discovered that genes that are critical for cell development are enriched within these variable lamina associated domains, when otherwise lamina proximal regions have been considered gene poor. This suggests that the nuclear periphery acts as an integral compartment in the nucleus that not only harbor repressed gene poor regions of the genome, but also acts as a critical level of regulation over genetic loci that are import for cell type specificity and development. Here in this work, we have discovered that DNA elements within LADs and components of the PRC2 complex establish *de novo* lamina associations. In addition, we suggest a role for levels of A-type lamins and, specifically, lamin C in regulating this repositioning which appears to be reliant of the chromatin modifications H3K27me3 and H3K9me2/3. In chapter 2 of this thesis, we used our TCIS system to identify sequences from fibroblast specific variable LADs containing the *Ikzf1* and *Bcl11a* loci. This work focused on LAD border regions due to the over representation in developmentally regulated genes. We show that these regions are sufficient for relocalization of an ectopic site to the nuclear lamina that re-organization is dependent on the PRC2 component YY1 and H3K27me3, a histone modification dependent on PRC2 and demonstrated to be enriched in these regions at endogenous LADs. In addition, we demonstrate, in agreement with two previous reports, that association with the nuclear lamina is also dependent on H3K9me2/3 (Bian et al., 2013; Towbin et al., 2012). This approach enabled the discovery of both cell-type specific (e.g. BTB/POZ domain proteins) and more ubiquitous components (CTCF and YY1) involved in scaffolding and maintaining segments of vLADs at the nuclear periphery. Recent reports have also identified specific nuclear envelop transmembrane proteins as involved in

organizing and regulating chromatin at the nuclear periphery (Reddy et al., 2008, Zuleger et al., 2013; and reviewed in Wong et al., 2014). We therefore propose a model for regulation of vLADs that incorporates cell type specific transcription factors along with chromatin modifiers and organizers to facilitate cell-type specific genome organization.

It will be of special interest to determine the underlying molecular machinery in establishment of broader lamina associated domains as well as the functional consequences of repositioning of a genic region to the nuclear lamina. Identification of specific factors that lead to LAD formation and delimiting of LAD boundaries will be of great import in understanding how organization at the lamina is influences development and how its dysregulation leads to disease. Additionally, our TCIS technology can be utilized to better understand the structure/functional relationships of not only the nuclear lamins as a functional sub-compartment but any nuclear compartment of interest. This technology will allow us to study in *vivo*, the epigenetic and macromolecular composition at a discrete DNA/chromatin segment in a manipulative manner.

Appendix I

Molecular interactions of proteins at the nuclear periphery,

BiFC: Bi- molecular fluorescence complementation and the nuclear lamina

When studying interactions of chromatin with the nuclear lamina we primarily relay on either cytological data or population based molecular assays (DNA methyltransferase identification, Dam-ID, through the utilization of a Lamin B1-Dam fusion protein). We were interested in methods that would detect molecular contact in single cells, such that they were more than just cytological and correlative. Most of the cytological data is scored by observing an overlap in flourophore signal, such as EGFP-LacI foci over lapping with an anti-lamin B1 stain, to denote the nuclear periphery. An overlap in these signals would be considered peripherally associated. DamID was also used in some cases to confirm molecular contact, though on a population basis. Bimolecular fluorescence complementation (BiFC) analysis allows for us to assay molecular contact of two nuclear regions in vivo and on a single cell basis (Figure AI.1). This technique was developed by Tom Kerppola as a means to study protein interactions in living cells (Kerppola, 2010; Hu et al., 2008). The assay is based upon the association of two non-florescent portions of a fluorescent protein, that when are brought in close, molecular proximity of each other by way of an interaction between proteins that are fused to each fragment, reconstitute fluorescence. This technology is beneficial in the study of any protein-protein interactions due to the ability to visualize these interactions inside a living cell. One can further identify differences among the population that would be drowned out in population based studies, and identify localization to sub-structures that otherwise would not be possible. Also, by assaying the interactions in living cells, one can avoid cell lysis and the possibility of missing the contents of the cell, which may lead to changes in protein interactions. One thing to note is that these experiments are done with exogenously expressed proteins and once the bi-molecular complex has formed, it is irreversible. Additionally, due to the time that is

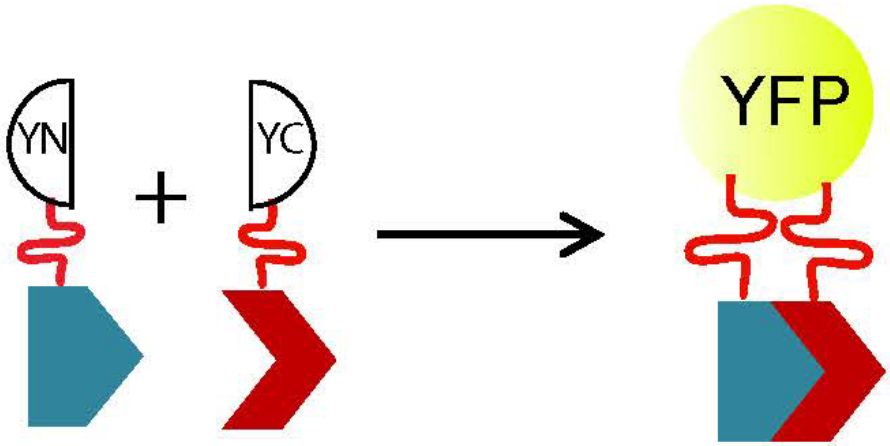


Figure AI.1: Bimolecular fluorescence complementation (BiFC). Schematic of the BiFC assay. Two non-fluorescent parts (YN and YC) of yellow fluorescent protein (YFP) are expressed as a fusion protein with possible interaction partners (Blue and Red shapes). Molecular association of the interaction partners leads to the formation of a full YFP that can then fluoresce.

required to form the active flourophore this assay is not a representation of what is happening in real-time. That being said, BiFC still is a powerful tool of visualization of protein-protein complex interactions with in live cells.

We utilized the BiFC approach to show molecular contact of a reorganized region of chromatin that has targeted to the nuclear periphery. Our utilization of the TCIS system for both random integration and discrete loci targeting studies made this possible through the *lacO*/LacI component of the system. TCIS is built upon two previously described technologies, Recombination Mediated Cassette Exchange (RMCE) and *lacO*/LacI (Feng et al., 1999; Belmont et al., 1999). The RMCE region makes it possible to directly integrate any DNA segment of interest into the TCIS system while the lacO/LacI system makes it possible to visualize the position of the locus in mammalian nuclei by the binding and accumulation of EGFP-LacI, to 256 *lacO* arrays. We designed a wide combination of the BiFC constructs by creating fusion proteins of each YFP non-fluorescent fragments, VN or YC to LacI at both the C and N terminus. Additionally, we created a variety of fusion proteins between inner nuclear membrane proteins and each YFP non-fluorescent fragments. We used LaminB1 and Lap2β initially. LaminB1 is a filamentous protein found underlying the inner nuclear membrane that gives structure and righty to the nucleus as well as has been shown to interact with IMN proteins, chromatin and various transcription factors with in mammalian cells. Lap2β is an INM, resident protein that has been implicated in interacting with repressed chromatin at the nuclear periphery (Zullo et al., 2012). The co-expression of a LacI- or INM- (LaminB1 or Lap2β) YFP fragments fusion proteins that would complement each other within our TCIS clone lines, would lead to the accumulation of LacI-VN/YC at the *lacO* arrays (in the absence of IPTG), while the LMN-YN/YC fusion protein would be expressed and targeted to the nuclear periphery where these endogenous proteins are found. In both

the Lap2β and Lamina fusions the BiFC fragment is inserted at the N-terminus of the proteins because in Lap2β the N-terminus is nucleoplasmic, while the C-terminus is not and for Lamins, the C-terminus cannot be blocked due to a farnesylation of a C-terminal CAAX box. In the presence of a fragments that has been integrated into the TCIS system and targets to the nuclear periphery we would expect that LacI-VN/YC would bind the *lacO* arrays, and after reorganization of the TCIS locus to the nuclear periphery, the close molecular proximity of the LacI-VN/Y and IMN-YN/YC proteins would lead to the formation of a functional fluorescent YFP proteins and thus visualization of the molecular interaction. We first validated these studies by visualizing the interactions of IMN proteins with themselves (known interactions) and recruitment of *lacO* to the nuclear periphery though random integration of peripheral targeting fragments and *lacO* array. Random integration of these fragments lead to high copy numbers of *lacO* arrays and the targeting fragments which are easier to visualize than the single TCIS locus alone, and these were done as first pass experiments.

Expression of BiFC constructs of LaminB1 and Lap2β show inner nuclear membrane proteins interactions (Figure AI.2). Co-expression of YN-LaminB1 and YC-LaminB1 and YN-Lap2β plus YC-Lap2β, show a faint but distinct rim staining and co-localizes with anti-LaminB1 staining. It is important to note is that these images were taken only 7 hours after transfection of the constructs into murine fibroblast cells. A further study of various time points after transfection, shows that overexpression of the exogenous proteins leads to perturbation of nuclear morphology, as seen by LaminB1 staining, the BiFC signal and overall DAPI staining (Figure AI.2). Initial studies concurrent with the development of BiFC examined the interaction of transcription

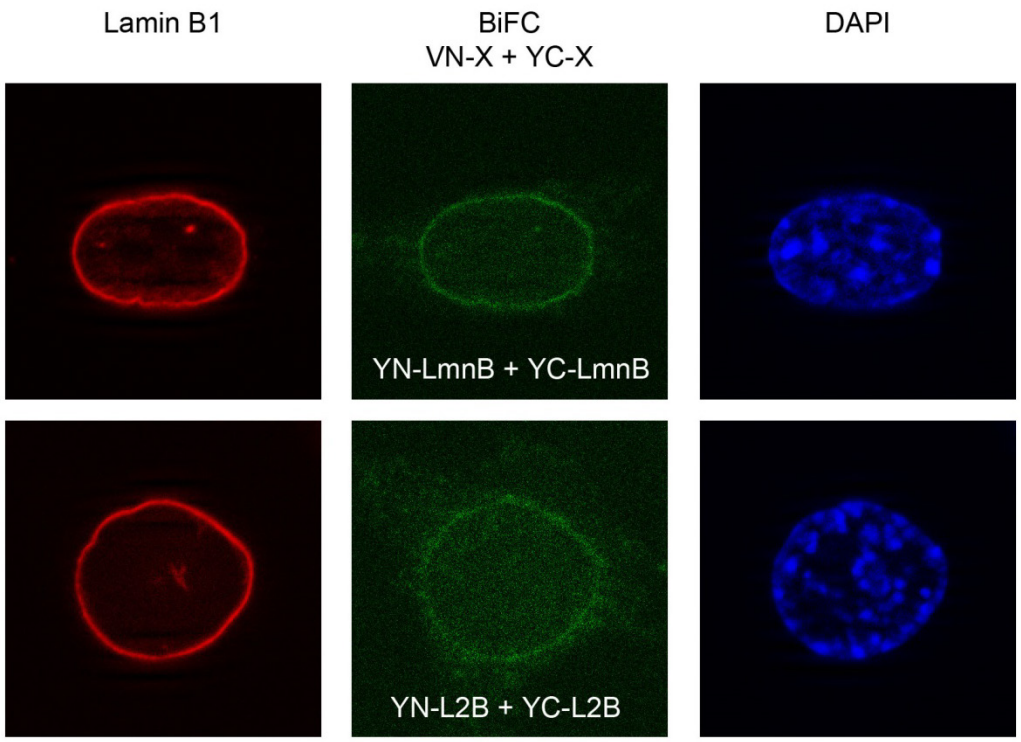


Figure AI.2: Expression of BiFC Lamin B1 and Lap2b fusion proteins show inner nuclear membrane (INM) protein interactions. Immunofluorescence was done in BI63T3 fibroblasts expressing YN- or YC- INM proteins, Lamin B1 (LmnB) or Lap2 (L2B). Images taken 7 hours after transfection.

factors, bZIP and Jun a Rel family protein (Hu et al., 2002). These studies allowed for visualization of the BiFC complex 18-48 hours after transfection. Early time points were avoided due to the lack of fluorescence seen with initial BiFC complex formation. We note that when studying proteins such as Lamin B1 and Lap2β within 14 hours there is a perturbation of nuclear architecture and therefore these results would be cofounded by perturbed conditions within the cell (Figure AI.3). These results none-the-less, confirm that our fusion construct produce a stable IMN proteins that will target to its resident sun-nuclear compartment, and self-interact as expected, to form a fluorescent YFP protein, thus showing functionality of the BiFC system.

To query the molecular contact of chromatin regions targeting to the nuclear periphery we utilized the *lacO*/LacI system and co-integration of *lacO* and fragmented Bacterial Artificial Chromosome (BAC) DNA identified to be in lamina associated domains (LADs) in murine fibroblasts. This DNA, which is found within LADs in fibroblasts, when randomly integrated into the genome of murine fibroblast, targets to the nuclear periphery, as done in previous studies (Zullo et al, 2012; Harr et al, 2015). In this case we co-express either YN/YC-LaminB1- or YN/YC-Lap2β with VN/YC-LacI in fibroblast cells harboring random integrations of BAC and *lacO* DNA. In the absence of IPTG the VN/YC-LacI fusion protein will bind the *lacO* arrays at the random integration site, and if these DNA fragments have targeted to the nuclear periphery we expect to see the formation of a fluorescent BiFC complex, as the *lacO*/LacI complex comes in close proximity to the nuclear periphery (Figure AI.4). As a verification of visualization, a positive control showing the interaction of VN-LacI and YC-LacI shows the random integration of the *lacO* and BAC DNA (Figure AI.4, panel A). Molecular contact of the BAC DNA targeting to the nuclear periphery is shown in Figure AI.4 panels B-D using

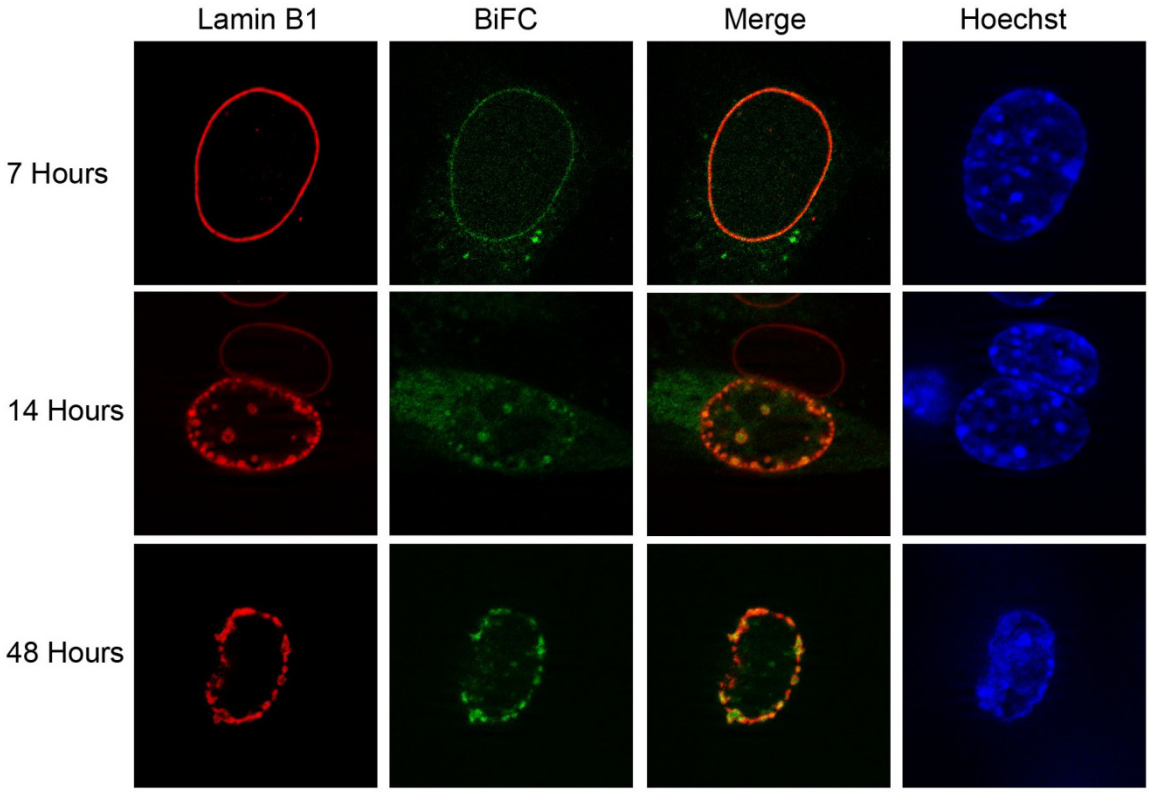


Figure AI.3: Expression of co-transfected YN-Lamin B1 and YC-Lamin B1 proteins leads to perturbation of nuclear morphology if expressed at higher levels and for too long. BiFC constructs for YN-LmnB1 and YC-LmnB1 are co-transfected into Bl6/3t3 Fibroblast cells. Cells are fixed at 7, 14 and 48 hours after transfection and stained for LmnB1 and Hoechst.

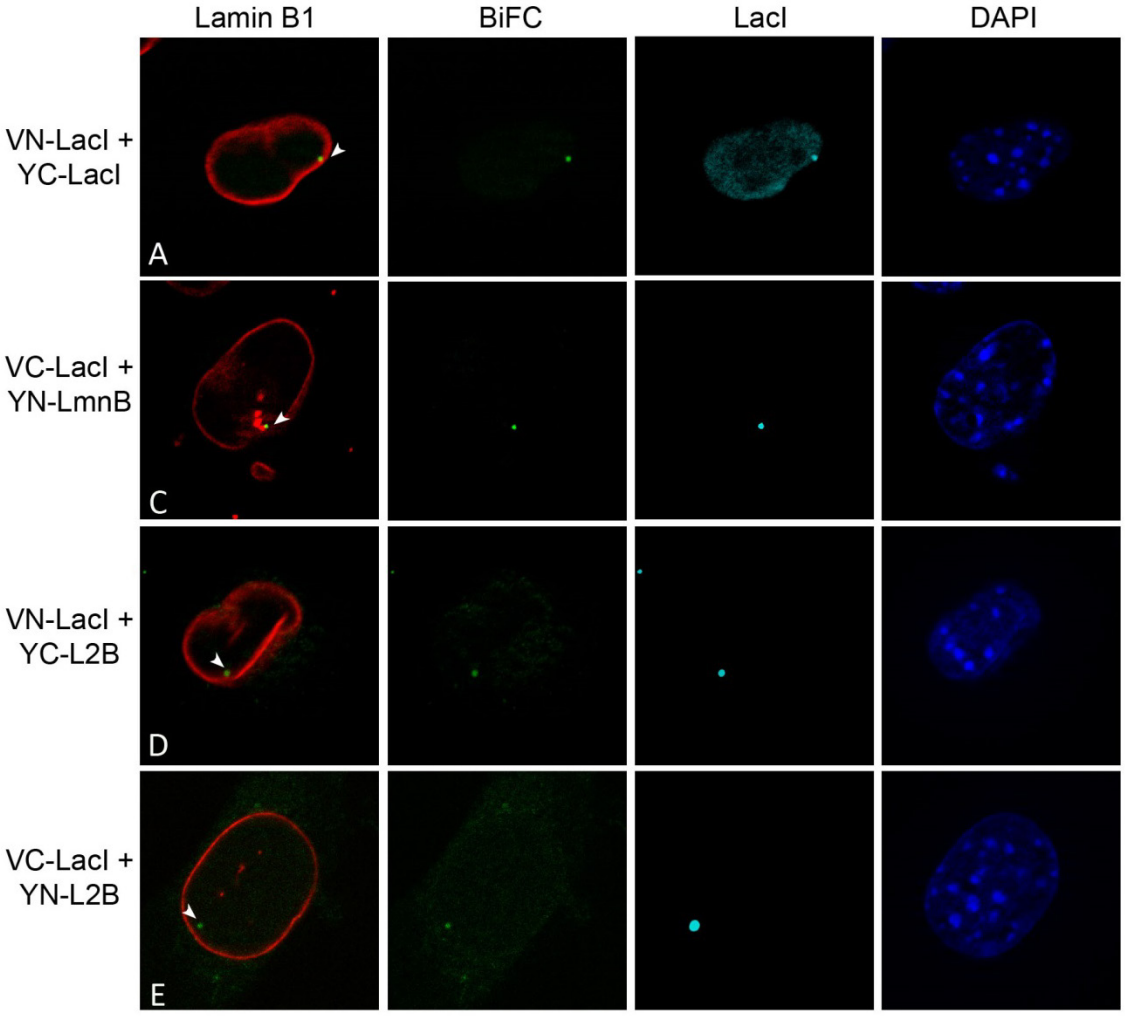


Figure AI.4: Co-expression of BiFC constructs show peripheral positioning and molecular contact (through BiFC) of LADed regions with the lamina and inner nuclear membrane proteins. A region of the Ikaros LAD encompassing BAC RP23-349M24 was randomly sheared and co-integrated with a *lacO* array in Bl6/3T3 fibroblasts. Panel A shows complementation of YN-LacI and YC-LacI at the integration site. Panels B-E show complementation of YN or YC-LacI with YN or YC-LaminB1 or Lap2b. Leftmost column shows both Lamin B1 staining (red) and complementation of BiFC fusion proteins (green). The second column depicts the fluorescence signal alone from the complementing proteins. The third and fourth columns are staining for LacI (cyan) and Hoechst (blue), respectively.

different configurations of the fusions proteins. A variety of DNA from fibroblast specific DNA was tested for its ability to target to the nuclear periphery, as well as a negative control of integration of *lacO* arrays alone (Figure AI.5).

The BiFC technique worked well to confirm the intra-molecular interaction of IMN proteins, LaminB1 and Lap2β with themselves, as well as show, *in vivo*, the molecular contact of a region of chromatin targeting to and residing at the nuclear periphery. This technique will prove difficult to carry out in single integration clones of the TCIS system, due to the faint levels of YFP expression upon complex of BiFC over background, at such low amounts as will be found at the contact sites.

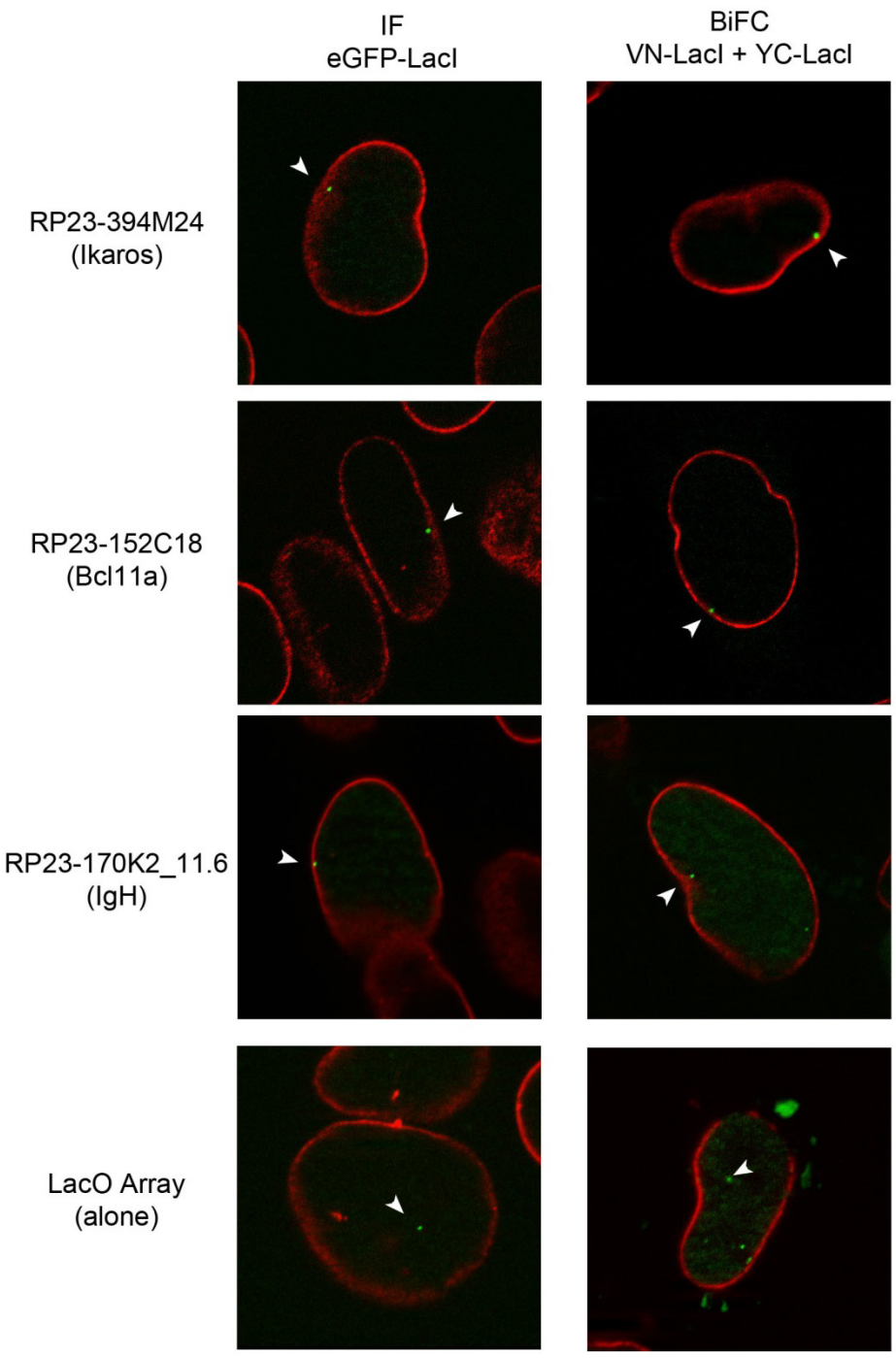


Figure AII.5: Random integration of LacO arrays and sheared BACs from *Ikaros, Bcl11a* and *IgH* Lamin Associated Domains into Bl6/3T3 fibroblasts direct peripheral association from ectopic sites in the genome. The left column shows eGFP-LacI (green) accumulation at *lacO* arrays. The right column shows Bi-Molecular Fluorescence Complementation of VN-LacI and YC-LacI accumulating at *lacO* arrays. Lamin B1 staining is in red.

Appendix II

Chap-MS: Determination of factors at a single mammalian locus

Every cell has the same genetic information (DNA), yet each cell, depending on its time or point in development will vary in phenotype and ability to interact with its extracellular environment. Each cell determines that part of its genome to appropriately express due to regulatory information within its epigenome. The epigenome includes chemical modifications to DNA (in the form of methylation) or post translational modifications (PTMs) to histones. With an advancing understanding of the influence of epigenetic factors and the proteins that play a role in controlling them, the underlying molecular mechanisms for this level of genetic control are poorly understood.

Chromatin Affinity Purification with Mass Spectrometry (ChAP-MS) is being combined with the TCIS System to investigate the role of factors that play a role in control over a single genetic locus. ChAP-MS was developed and first utilized in S. *cerevisiae* where Byrum et al. specifically enriched for a ~1,000 base-pair section of GAL1 chromatin under transcriptionally active and repressive conditions. After enriching for a small region of DNA in two different states they further identified the specifically bound proteins and histone posttranslational modifications (Byrum at al. 2012). This technique yields much more information than traditional chromatin immunoprecipitation (ChIP), due to its ability to query the nuclear environment without bias. ChIP is limited in its ability to assay for the proteome and histone modifications due to the lack of specific antibodies, as well as relying on their use (a biased probe). Additionally, it can lead to the identification of combinatorial histone modifications on a single histone as obtained from a native environment, something that was not able to be done before. ChAP-MS allows for the isolation of a native genomic locus that is then assayed by high resolution proteomic identification by mass spectroscopy, to identify without bias, any interacting proteins and posttranslational modifications.

To assay these questions in mammalian cells we need to be able to integrate the ChAP-MS constructs into a single genic location. The TCIS system is well established in

murine fibroblast cells and has been used to study the role of reorganization of chromatin to the nuclear periphery (Harr et al., 2015). These established cell lines are being used to integrate ChAP-MS constructs at a single mammalian locus. By Cre-recombinase mediated recombination, the TCIS site can accept a DNA construct containing the components required for ChAP-MS. With the inclusion of a test gene (GFP) driven by an inducible promoter we can assay the effect of gene modulation on macromolecular chromatin composition *in vivo*, in mammalian cells (Figure AII.1 A/B). Additionally, through the *lacO* arrays of the TCIS system, we can also recruit chromatin modifiers to assay the effects on macromolecular chromatin composition (Figure AII.1 C). Furthermore we can elaborate on previous work carried out in TCIS. TCIS system has recently been utilized to recruit a discrete genomic region to the nuclear periphery through either the integration of a targeting cis element, or targeting of a specific protein (YY1). By introducing the ChAP-MS construct into existing TCIS cell lines that have previously been queried for their ability to re-organize chromatin; we can further refine and understand the protein players and changes in chromatin composition (modifications) with recruitment to the nuclear periphery (Figure AII.1 D). One can image this could be adapted to determine macromolecular chromatin composition and consequences of targeting to any compartment with in the nucleus with the discovery of new targeting *cis* or *trans* elements.

Figure AI.1: Integration of ChAP-MS in mammalian cells via TCIS technology and schematics for proposed experiments. (A) A ChAP-MS construct containing a LexA or TALEN protein binding domain, and an enhancer/promoter region driving a destabilized GFP reporter gene. This construct is cloned inside inverted LoxP sites in a "switch" vector. Expression of this vector in the presence of Cre recombinase in a cell line containing the TCIS integration will lead to recombination mediated cassette exchange and insertion of the ChAP-MS construct at a single integration site. The ChAP-ME technology once integrated into mammalian cells can be used in multiple ways to assay macromolecular protein composition at a single locus *in vivo*. In each of these scenarios, material will be processed by ChIP and subsequent Mass-Spectroscopy analysis. (B) Investigation of the macromolecular protein composition before and after gene induction by an inducible promoter. (C) Investigation of the macromolecular protein composition before and after recruitment of histone modifying proteins (HMP) to the locus through either the LexA/TALEN binding sites or *lacO* arrays. (D) Investigation of the macromolecular protein composition before and after reorganization of a discrete locus. This can be done through the use of either a targeting DNA element or recruitment of a protein to the either the LexA/TALEN binding sites or *lacO* arrays, that would lead to an association with a nuclear sub-compartment.

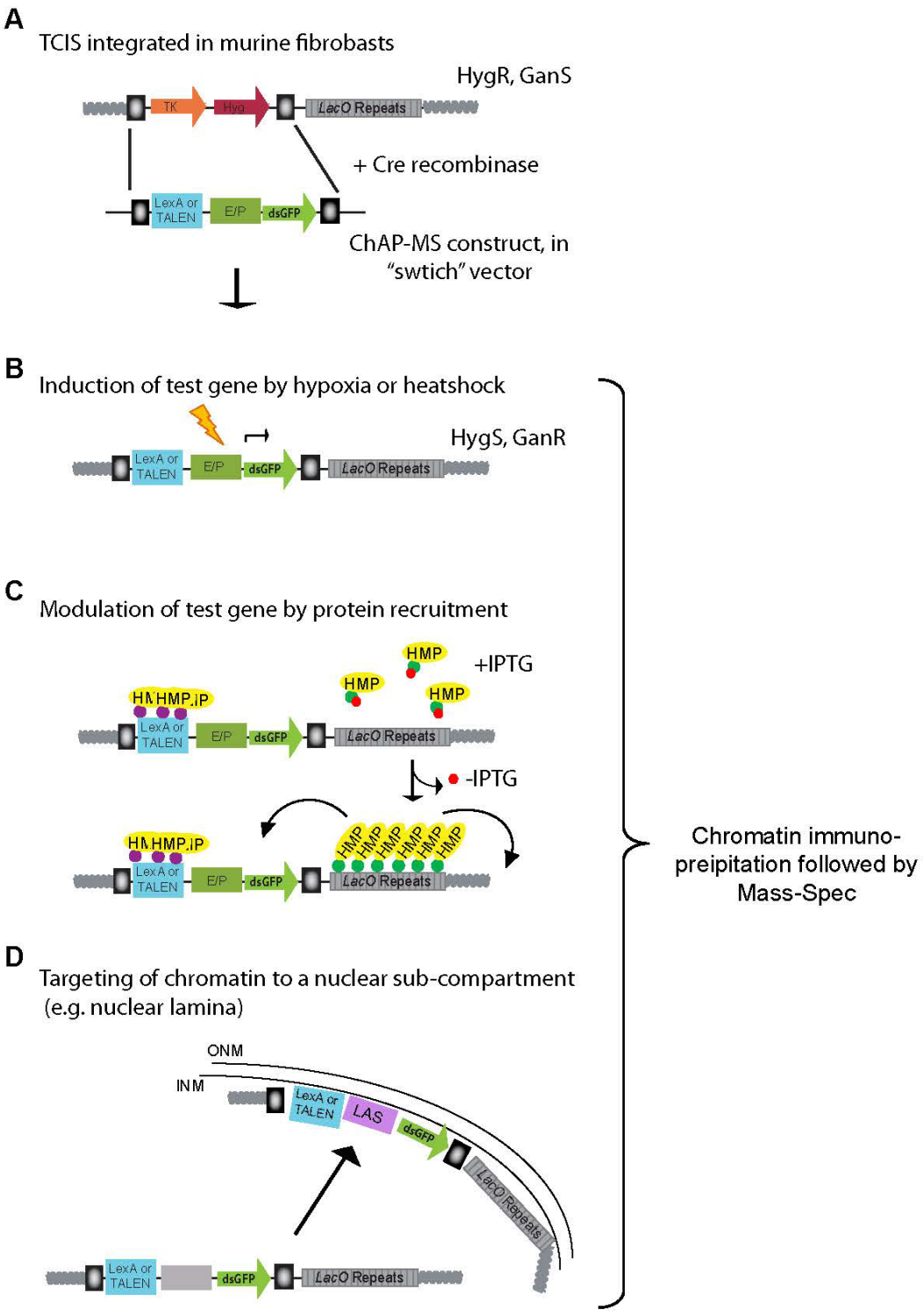


Figure AII.1: Integration of ChAP-MS in mammalian cells via TCIS technology and schematics for proposed experiments.

Experimental Plan

Overview

This project will start with the development of constructs (Figure AII.2 A) followed by validation by testing the ability to pull down a locus with the LexA-PrA handles. Next we will establishment of a method for growing large enough cultures for ChAP-MS: To carry out ChIP on a single locus in a mammalian cell, cells will be needed in the range 10^7-10^{10} cells/condition. The Chap-MS construct will then be incorporated into murine mammalian cells though site directed recombination into established TCIS Clone lines Y and 12. After manipulation of experimental conditions (hypoxia/ heat shock/ nuclear compartmentalization or recruitment of HMP) cells will go through an established ChIP protocol (Byrum et al., 2010) followed by Identification of macromolecular protein complexes and PTMs by mass spectroscopy.

Creation of the ChAP-MS-TCIS constructs and establishment in mammalian cells:

The LexA binding site was engineered immediately upstream of an inducible enhancer/promotor (hypoxia inducible element OR heat shock element) flowed by a minimal promoter, to drive the expression of a destabilized GFP. The LexA DNA binding site will interact with a constitutively expressed LexA-Protein A (LexA-PrA) fusion protein, that can be used as a protein "handle" to affinity purify our chromatin region of interest. The destabilized GFP contains a PEST domain that targets it to the proteasome for rapid degradation, allowing for real time analysis of the expression of a test gene at our locus, by flow cytometry or microscopy (Salama et al., 1994; Belizario et al., 2008). Additionally with an inducible gene was can study either active genes (under induced

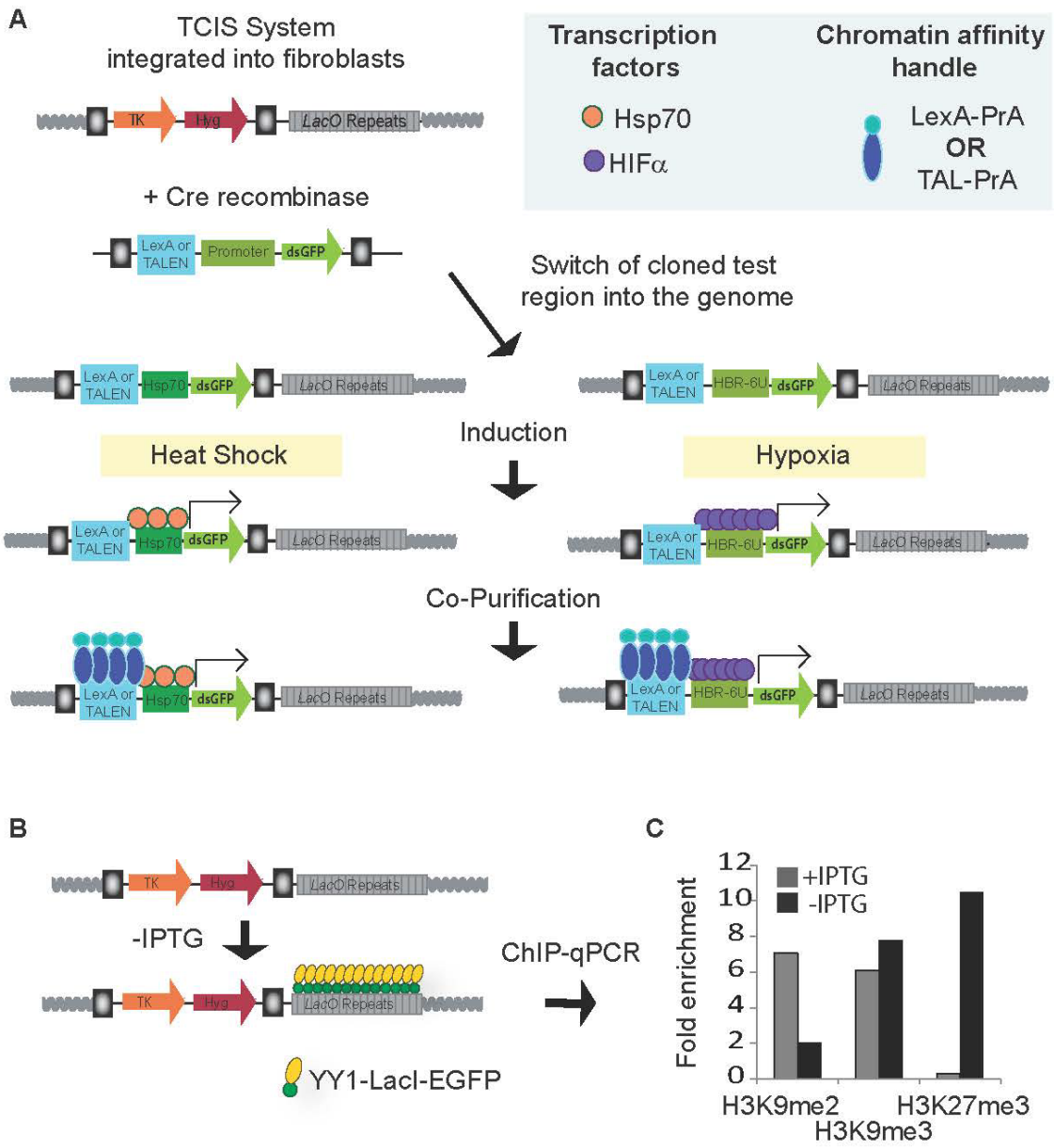


Figure AII.2: Adaptation of ChAP-MS to inducible mammalian promoters. (A) Schematic of the TCIS System and; integration of cloned test region having an inducible promoters which containing 6 or 12 binding sites for either LexA or TAL, and which binding sites directing expression of a destabilized dsRED gene. Hsp70: heat shock protein 70, HBR-6U: 6 hypoxia- inducible repeats from the HIF1α promoter. (B) Left, schematic showing recruitment of YY1 to a distinct locus. Right, ChIP-QPCR qPCR of locus after pull-down with α-H3K9me2, α-H3K9me3, or α-H3K27me3, as indicated showing YY1-induced enrichment of H3K27me3.

conditions) or inactive gene. This engineered region will be incorporated into a 'switch' cassette vector, which contains the inverted LoxP (L1) elements. These L1 elements will flank the engineered region, allowing for incorporation of the inducible E/P-LexA-DsGFP into the TCIS system (Figure AII.A).

To recruit histone modifying proteins (HMPs) to our locus, fusion proteins will be created with a protein of interest and either LexA/TALENs of LacI. The inclusion of a GFP in the fusion will allow for the ability to visualize the accumulation of protein X-EGFP-LacI (LexA) at the TCIS locus and in live cells, visualize any nuclear compartmentalization or movements that may take place. We will test HMPs that have the potential to alter the epigenome. For active genes (under induced conditions) we will recruit HMPs that can lead to repression, such as; G9a, Suv39H2, PRDM, HDAC1 and HDAC3. To inactive genes (permissive conditions) we will recruit HATs and HMPs that are known to lead to gene activation such as; p300, MLL3/4 and UTX). It is expected that by recruitment leading to a change in gene expression we will be able to alter the chromatin state of the TCIS locus as assayed by real-time PCR and ChIP.

Downstream analysis:

After setting up the TCIS-ChAP-MS system in mammalian cells, experimental cells will be fixated and chromatin affinity purification will be carried out with conditions already optimized for yeast (Byrum et al., 2012). LexA-PrA enrichment of the E/P elements will initially be determined by ChIP followed by quantitative PCR using primers to the engineered promoter regions. We will explore enrichment of 1kb sections as well as larger fragments. Proteins co-enriching with LexA-PrA will then be determined by I-DIRT (Smart et al., 2009; Byrum et al., 2012). I-DIRT (Isotopic differentiation of interactions as random or targeted), is a technique the makes it possible to differentiate between specific and nonspecific proteins in a complex that has been isolated by ChIP

(Tackett et al., 2005). A major hurdle with using mass-spectroscopy to identify proteins with in complexes isolated from mammalian systems is the presence of nonspecific proteins. This technique uses isotopic labeling to label specific proteins interactors and at the analysis step, determine which proteins are contaminants and not part of the complex due to differential isotopic labeling. In our system we expect enrichment of the promoter elements only in cells where both LexA-PrA and the E/P-LexA-DsRed are both present.

Summary

This project will shed light on to the mechanisms behind epigenetic regulation at a specific locus in mammalian cells. It will lead to the identification of proteins involved in this process as well as give an unbiased view of the chromatin state before and after locus manipulation by either gene induction, histone modification (though directed targeting of histone modifying proteins) and change in nuclear environment my directed targeting of the locus to a nuclear sub-compartment (e.g. the nuclear periphery). This work leads to the generation of new methodologies to manipulate and study the epigenome, and therefore broaden the understanding of regulatory pathways controlling chromatin.

Appendix III

The distinct roles of Lamin A and Lamina C in genome organization

Our studies of identifying cis and trans factors involved in reorganization of chromatin to the nuclear periphery revealed a role for YY1, chromatin modifications, H3K27me3, H3K9me2/3 and architectural a proteins including CTCF, and Lamin A/C (Harr et al., 2015). We hypothesize that lamin A/C may be helping anchor or maintain chromatin at the periphery once it has reached this nuclear sub-compartment. An earlier study (in collaboration with our lab) had implicated Lap2β, an inner nuclear membrane (INM) protein as being involved in this process, though we were unable to verify this in our system (Zullo et al, 2008). We choose to investigate the role of the lamins in this process due to their prominent location at the nuclear periphery. Lamins are intermediate filament proteins that assemble as a fibrous structure up against the inner nuclear membrane. In this position they play a role of providing nuclear structure and have been shown to interact with chromatin. The Lamina network includes two major classes, the A-type and B-type lamins (Gerace and Berk, 1988; Stuurman et al., 1998; Moir et al., 1994). B-type lamins include lamin B1 and lamin B2, each of which are produced from different genes (Höger et al., 1990; Weber et al. 1990). On the other hand, the two major isoforms of A-type lamins in mammals are lamins A and C, which are generated from the game gene, *LMNA* (Fisher et al., 1986; McKeon et al., 1986).

Lamin A and Lamin C are the same for the first 566 amino acids, yet differ at the C-terminal domain. Lamin A is 72 kDa and is produced by an alternate 5' splice site with in exon 10 that includes two more exons than lamin C. Lamin C is 65 kDa, and it is produced from a transcript composed of exons 1-10. This results a unique C terminus for each lamina A and C, lamin A having 98 unique amino acids and lamin C had 6 unique amino acids in this region (Al-Saaidi and Bross, 2014). This variation on the amino acid sequences leads to lamin C being left as it is, while lamin A is further processed such that the carboxyl- terminal CaaX sequence it is processed with leads to a post

translational modification that produced mature lamin A. This multi-part process involves the farnesylation of cysteine (by farnesyltransferase) followed by the removal of the last three amino acids by ZMPSTE5, and lastly carboxy-methylation of the C-terminal farnesylcysteine by isoprenylcysteine carboxyl methyltransferase Some evidence is emerging showing that these proteins play distinct biological roles (see review; Al-Saaidi and Bross, 2014; Zwerger and Medalia, 2013).

The nuclear periphery is being highly studied for its roles in gene regulation and a better understanding as to why specific regions of the genome reside within it, as a sub-compartment. Cytological and genome-wide studies have shown that the nuclear periphery contains large lamins associated domains (LADs) of chromatin that are either gene poor, or that have an over representation of developmentally regulated genes that when in this compartment are repressed. The lamins are believed to play an important role in development and disease due to the occurrence of a variety of developmental disorders that arise from mutations in the proteins of the nuclear lamina (Nagano and Arahata, 2000; Worman, 2012). We are investigating the role of A-type lamins in either helping with recruitment or maintenance *de novo* LAD formation. At this time of the study we had available to us knockdowns for the *LMNA* gene that lead to reduction in both lamin A and lamin C proteins (Harr et al., 2015). Specific roles for lamin A and C have not been highly vetted due to the lack of tools to study their differences in mammalian cells. In the Reddy lab, Xianrong Wong created highly specific shRNA targets that result in the reduction of either lamin A or lamin C. This gives us the unique opportunity to investigate is these isoforms played a different role in maintenance of de novo LADs at the nuclear periphery.

Recent studies have implicated lamin A in large scale chromatin organization, but they were not able to differentiate between the role of lamin A and C (McCord et al., 2013; Solovei et al., 2013). We showed that either overall levels of lamin A/C are

involved genome organization, suggesting that lamin C is playing a role due to the clear results we obtained showing that lamin A is not necessary for recruitment of retention of chromatin at the nuclear periphery. This suggests, contrary to the commonly held position that there is functional redundancy between lamina A and lamin C, that lamin C may be specifically involved in instructing peripheral positioning. Consistent with this, are studies that "lamin C-only" mice (missing lamin A but with normal lamin C levels) are viable, yet LMNA/C$^{+/-}$ have perturbed differentiation (Fong et al., 2006; Sehgal et al., 2013).

In further support of lamin C playing a distinct role in organization of chromatin at the nuclear periphery we utilized a lamin C specific knockdown construct to investigate how lamin C plays a role in establishment and maintenance of peripheral associating chromatin. Reduction in lamin C levels in TCIS fibroblast clones harboring either a peripherally targeting DNA fragment or YY1-EGFP-LacI proteins that targets to the nuclear periphery, we see that there is a loss of association and maintenance of TCIS *de novo* LADs. This is in contrast to lamin A specific knockdowns, that to not perturb the peripheral association of these lamina associating regions (Figure AIII.1). Additionally, endogenous loci present in variable lamina associated regions containing the developmentally regulated genes; *Igh, Ikaros* and *bcl11a*, and that have all been shown to have differential lamina association dependent on their transcriptional status in the cell, also show a lamin C dependent loss in peripheral association (Figure AIII.2).

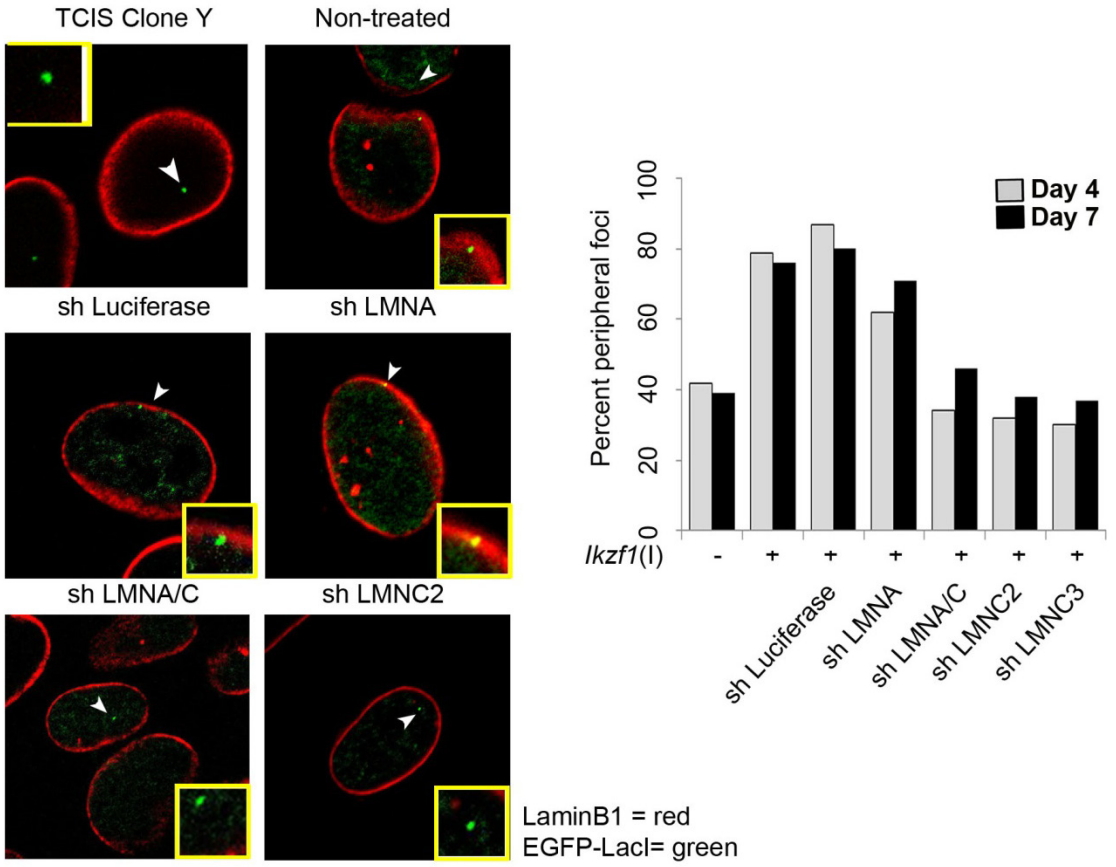


Figure AIII.1: Lamin C and not Lamin A is involved in *de novo* LAD organization and/or maintenance. Left, Representative immunofluorescence images of fibroblast TCIS clone Y. TCIS clone Y harboring a peripheral targeting fragment (I) from the fibroblast vLAD containing *Ikzf1*, was treated with shRNA for luciferase (off-target control), Lamin A or Lamin C (two different shRNA targets). Pop out is 300X. Green= EGFP-LacI at *lacO* array, red= anti-LaminB1. Array indicates TCIS locus. Right, Quantitation of immunofluorescence in two TCIS clone lines (Y and 12). n≥ 50.

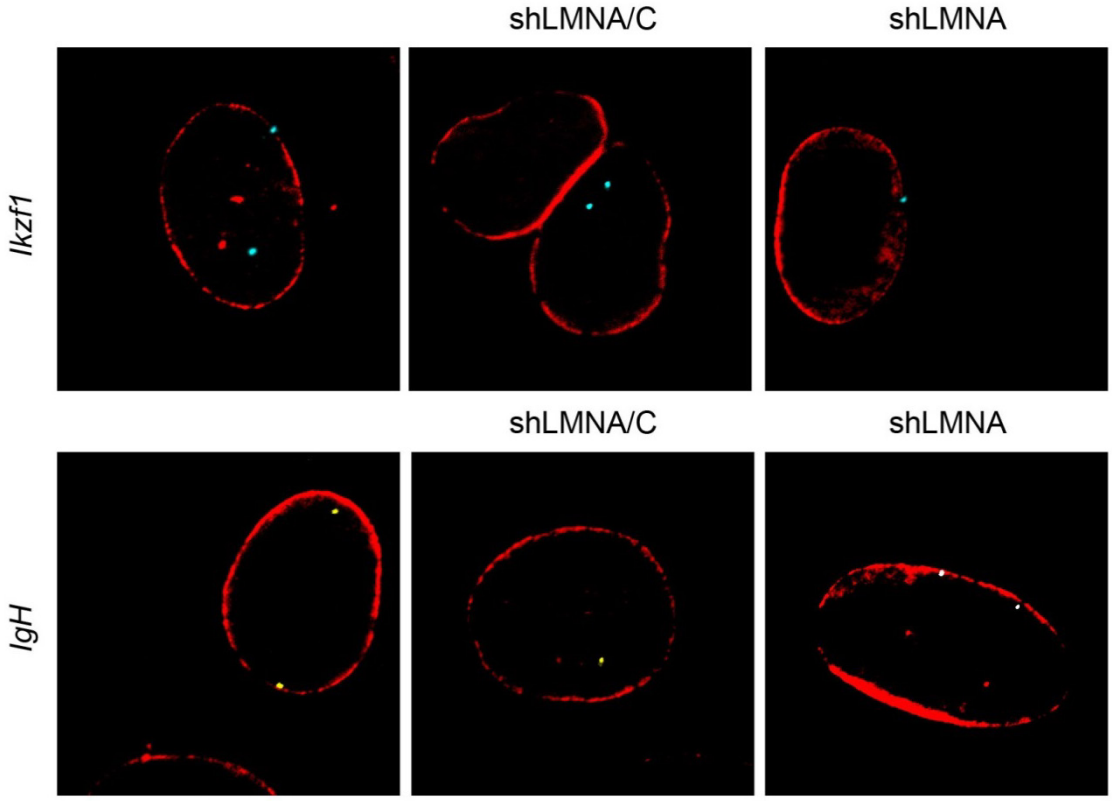


Figure AIII.2: Knockdown of Lamin A/C and not Lamin A alone reduces peripheral association of *Ikzf1* and *IgH* endogenous loci in mouse embryonic fibroblasts. Representative images of fluorescence *in situ* hybridization with a DNA probe to either the *Ikzf1* or *IgH* locus in mouse embryonic fibroblast cells (Cyan and yellow, respectively). Red, anti-Lamin B1.

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University of the Pacific, Stockton, CA **2005-2007**
M.S., Pharmaceutical and Chemical Sciences.

Sonoma State University, Rohnert Park, CA **2001-2005**
B.S., Molecular and Cellular Biology, with a minor in Chemistry.

Denmark's International Study Program, Copenhagen, Denmark **2004**
Study Abroad, toward completion of B.S.

RESEARCH EXPERIENCE

Johns Hopkins School of Medicine, Baltimore, MD (2010-2015)
Center for Epigenetics and Department of Medicine
Ph.D. Mentor: Dr. Karen L. Reddy

- Discovered cis sequences, protein players and a proposed a mechanism for the establishment and mechanism of variable lamina associated domains (LADs) in mammalian cells as a way to regulate the expression of key genes in development and differentiation. The resulting publication is the first to show of a role for YY1 and A-type lamins in LAD formation and maintenance

- Developed a novel tool to study ectopic sequences and to visualize them at the single cell level without the consequences of position effect and multiple integrate copies.

University of the Pacific, Stockton, CA (2005-2007)
M.S. Mentor: Robert F. Halliwell

- Investigated the role of natural grape compounds as modulators of the GABAA receptor. Resveratrol and Catechin were shown to have highly specific modulatory roles at distinct GABAA receptor subtypes.

- Studied the effect of environmental enrichment on the quality of Xenopus Laevis oocytes for use in two-electrode voltage clamp electrophysiology.

Sonoma State University, Rohnert Park, CA (2001-2005)
Mentor: Carmen Works

- Bioinorganic Chemistry laboratory, studied the photochemistry of Fe-only hydrogenase model compounds.

ACADEMIC EMPLOYMENT

Adjunct Faculty **Spring/2014-Spring/2015**
Towson University, Towson MD

Course instructor for Bio120, Biology for non-majors

- Developed, led and instructed a required lecture course of biology for non-majors

Research Specialist **Apr/2009-Aug/2010**
Johns Hopkins University, Baltimore, MD

Research was carried out under the direction of Karen Reddy, Ph.D. in the Department of Biological Chemistry.

- Developed and carried out experiments to investigate the function of the nuclear periphery as a distinct nuclear compartment and its effect on gene expression.

Research Specialist **Feb/2008-Feb/2009**
Johns Hopkins University, Baltimore, MD

Research was carried out under the direction of Baogen Su, Ph.D. and Nicholas Flavahan, Ph.D. in the department of Anesthesiology and Critical Care Medicine.

- Developed and executed experiments pertaining to the signaling pathways involved in the development of small arteries and veins in the cardiovascular system.

Teaching Assistant **Aug/2007-Dec/2007**
University of the Pacific, Stockton, CA

Graduate students led laboratory and discussion sections for pharmacy students attending the University of the Pacific.

- Lead laboratory and discussion sessions for over 80 pharmacy students.
- Worked with pharmacy professors to develop and write labs.

TEAM Science Fellow **Aug/2005-Jul/2007**
University of the Pacific, Stockton, CA: GK-12 Grant Graduate Student Fellowship Program

The National Science Foundation GK-12 grant was awarded to the University of the Pacific to teach and introduce science to elementary school students in the community. In this role, appointed graduate students at the University of the Pacific have the responsibility to introduce basic science in the field of biology, chemistry, physical and earth science to grade school students in San Joaquin County.

- Developed and lead inquiry based science experiments with 6^{th}-12^{th} grade student classrooms.
- Worked directly with teachers to develop and improve their abilities in teaching science.
- Worked with students to plan and carry out a yearlong science project.

Supplemental Instructor **Aug/2003-Dec/2003**
General Sciences Tutor **Aug/2002-May/2003**
Tutorial Center: Sonoma State University. Rohnert Park, CA

The SSU tutorial center provides free tutoring to students. The tutorial center employs senior students as supplemental instructors to lower level undergraduates.

- Created lesson plans for a group of 20-50 general chemistry students to help increase their study skills and assist them in succeeding in the course.
- Worked with the general chemistry course coordinator to plan out supplemental lesson plans.
- Assisted in writing and proctoring examinations.
- In the capacity of science tutor, I worked to assist students in biology and chemistry.

INDUSTRIAL EMPLOYMENT

Laboratory Technician **Jan/2005-May/2005**
Medlogics Device Corporation, Santa Rosa, CA

Medlogics is a small biotech with a fully operational R&D laboratory, with the goal of developing drug releasing medical devices.

- Assisted in preparing the new clean analytical chemistry laboratory for GLP compliance.

- Prepared samples of drug coated stents and analyzed samples by HPLC.

PROFESSIONAL MEMBERSHIPS

American Society for Cell Biology
Rho-Chi Pharmacy Honor Society
Tri-Beta Biological Honor Society
American Chemical Society

AWARDS, HONORS AND SCHOLORSHIPS

- Scholarship to attend CSHL Nuclear Organization and Function 2014 Meeting
- Towson University/JHU PDO Teaching Fellow, 2014
- Life time member of Rho-Chi Pharmaceutical Honor Society, 2007-present
- Recipient of full scholarship to attend the Cold Spring Harbor Course: Molecular and Cellular Biology of *Xenopus*, April 2007
- First place in the MicroMed Laboratories research project, 2005
- Recipient of Tri-Beta Biological Honor Society research grant, 2004
- Life time member of Tri-Beta Biological Honor Society, 2001-Present
- Recipient of Sonoma State University Dean's List/Honors, 2001-2005
- Recipient of the National Merit Scholarship, 2001-2005
- Recipient of the Golden State Scholar Share-governor's scholarship program, 2001
- Awarded the Sonoma State University President Scholarship, 2001
- Awarded the Loys T. Newton Scholarship, Scottish Rite, 2001
- California Scholarship Federation Member, 1998-2001

PUBLICATIONS

Invited / in preparation

Harr, J.C., Wong X., Reddy K.L. (*invited, 2015*) **"**Targeting chromatin to the nuclear lamina." *Nucleus.*

Harr, J.C. and Reddy, K.L. (*invited, 2015*) "A new perspective on the nuclear periphery." *BioEssays*

Harr, J.C. and Reddy K.L. (*invited, Jan 2016 issue*) Tagged Chromosomal Insertion Site (TCIS): A system for live cell imaging and tracking of a single element without position effect. *Methods in Enzymology*

Published

Harr, J.C., Luperchio, T.R., Wong X., Cohen, E., Wheelan, S., Reddy, K. (2015) Directed targeting of chromatin to the nuclear lamina is mediated by chromatin state and A-type lamins". *The Journal of Cell Biology* 208(1) 33-52.

This work is highlighted in a biobytes podcast, a Johns Hopkins SOM press release and in a Research Highlight (Nature Reviews Molecular Cell Biology 16, 68 (2015) doi:10.1038/nrm3948)

Harr J.C., and Reddy K.L. (2013) Live Cell Imaging of Nuclear Dynamics. In: Lennarz W.J. and Lane M.D. (eds.) *The Encyclopedia of Biological Chemistry*, vol. 2, pp. 749-755. Waltham, MA: Academic Press.

Zhu, J.-H., Chen, C.-L., Flavahan, S., **Harr, J.**, Su, B., & Flavahan, N. A. (2011) Cyclic stretch stimulates vascular smooth muscle cell alignment by redox-dependent activation of Notch3. *American journal of physiology Heart and circulatory physiology*, *300*(5), H1770–H1780.

Harr J, Coyne L, Chandry A, Halliwell RF. (2008) An Electrophysiological Study of the Impact of Environmental Enrichment on Xenopus Laevis Oocytes. *Animal Welfare Institute Quarterly*, vol. 57(3)

Harr J. (2007) An Electrophysiological Study of the Effects of Resveratrol and Catechin at GABAa Receptors**.** Thesis [M.S.] University of the Pacific

ABSTRACTS/PRESENTATIONS

Harr J.C., Reddy, K. *Directed reorganization of chromatin to the nuclear lamina is mediated by chromatin state, YY1 and A-type lamins*. Talk, ASCB, December 2014

Wong, X. **Harr, J.C.**, Reddy, K.R. *The Role of A Type Lamins in Genome Organization*. Abs, ASCB December 2014

Harr, J.C., Luperchio, T.R., Wong X., Cohen, E., Wheelan, S., Reddy, K. *Directed reorganization of chromatin to the nuclear lamina is mediated by YY1*. Abs, CSHL Nuclear Organization and Function, August 2014

Harr J, Zhu J-H, Chen, C-L, Flavahan NA, Su, BY. *Redox regulation of tissue transglutaminase contributes to mechanosensitivity of vascular smooth muscle cells.* ACCM Research Day, Abs. 2008

Harr J, Payne T, Basi R, Blankenship J, Jones P. *TEAM Science; Teaching, Experimenting and Mentoring in Science. ACS,* Abs. 2006

Harr J, Martineau N, Works C. *Photochemical Studies of a Model Compound for Iron-only- Hydrogenase.* ACS, Abs. 2005